ACTIVATION, REGULATION AND FUNCTIONAL CHARACTERIZATION OF CLASS II PI3KC2B

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ABBREVIATIONS

ACK1	activated Cdc42Hs-associated kinase 1
Akt/PKB	murine thymoma viral oncogene homolog 1/ Protein Kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
CHIP	carboxyl terminus of Hsc70-interacting protein
c-Kit/SCFR	mast/stem cell growth factor receptor
c-Met	hepatocyte growth factor receptor
Dbl	diffuse B-cell lymphoma
DH	Dbl-homology domain
DN	dominant-negative
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
Erk	extracellular signal-regulated kinase
ESCC	oesophageal squamous carcinoma
FGFR	fibroblast growth factor receptor
GAP	GTPase activating proteins
GBM	glioblastoma multiform
GDI	guanine nucleotide dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
GTPase	guanosine triphosphate phosphohydrolase
Hsc70	heat shock cognate protein Hsc70
Hsp90	heat shock protein Hsp90
IGF-IR	insulin-like growth factor receptor
INTS	intersectin
IR	insulin receptor
JNK/SAPK	c-Jun N-terminal kinase/stress actvated protein kinase
KD	kinase-dead
LC-MS	Liquid chromatography-mass spectrometry

LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MII	myosin II
MTM1	myotubularin 1
mTORC1	mammalian target of rapamacin-raptor complex 1
mTORC2	mammalian target of rapamacin-rictor complex 2
NB	neuroblastoma
NPDL	nodular poorly differentiated lymphoma
NSCLC	non-small cell lung cancer
onco-Dbl	oncogenic Dbl
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase-1
PH	Pleckstrin-homology domain
PI3K	Phosphatidylinositol-3 kinase
PI3KC2β	Phosphatidylinositol 3-kinase C2 domain containing subunit beta
PNET	peripheral neuroectodermal tumor
proto-Dbl	prototype Dbl
PTB	phosphotyrosine-binding
PtdIns	phosphatidylinositol
PtdIns(3)P	PtdIns 3-phosphate
PtdIns(3,4)P_2	PtdIns 3,4-bisphosphate
PtdIns(3,4,5)P_3	PtdIns 3,4,5-trisphosphate
PtdIns(4,5)P_2	PtdIns 4,5-bisphosphate
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RTK	receptor tyrosine kinase
S6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase 1
SCF	stem cell factor
SCLC	small cell lung cancer
SH2	Src homology-2
SH3	Src homology-3
SHIP	SH2-containing phosphatase
SNP	single-nucleotide polymorphism
Sos	son of sevenless
WT	wild-type

SUMMARY

Class II PI3K (phosphoinositide 3-kinase) isoforms have not been extensively investigated since the whole family of PI3Ks was discovered in the 1980s, and class II PI3K family members are still the least studied among all PI3Ks. To date the interest of the scientific community was mostly focused on class I PI3K enzymes especially due to their well established role in the development of several human disease including diabetes and cancer. However, an increasing amount of data has emerged recently suggesting an important role of class II PI3Ks in physiological and pathological processes. Together with receiving more attention, the understanding of their cellular functions will considerably increase. There is not much known yet about the mechanisms of class II PI3Ks activation and their downstream targets. Interestingly, accumulating data in the literature suggest that due to synthesis of distinct phosphoinositide or through different intracellular localization than other PI3K isoforms, class II PI3Ks possibly regulate biological processes or different steps in the same process distinct from class I and III PI3Ks. The molecular mechanisms underlying their action however still remain unrevealed.

PI3KC2β belongs to class II PI3Ks and its cellular functions have been associated with pro-migratory and pro-survival signals, as well as cell proliferation. Following ligand stimulation, the enzyme is recruited to the activated receptor tyrosine kinases (RTKs) via Grb2 or Shc adaptor proteins. At the plasma membrane PI3KC2β generates mostly PtdIns(3)P or PtdIns(3,4)P_2 and forms multi-protein complexes, whose assembly lead to activation of the Rho GTPases and regulation of the Akt/PKB signaling pathway. How exactly PI3KC2β contributes to the above-mentioned functions has not been precisely described. However, its mechanism of action seems to be related to the kinase multi-domain structure, which differs much from the structures of class I and class III PI3Ks. The most characteristic features of the enzyme are its lack of association to regulatory subunits, which is accompanied by a high molecular mass and elongated N- and C-terminal extensions, which play a regulatory role for PI3KC2β catalytic activity. The knowledge about PI3KC2β structural features still needs to be translated into a precise mode of action.

In my studies I have investigated the regulatory mechanism linking PI3KC2β to the activation of the Rho family of small GTPases through the Dbl guanine exchange factor (GEF) in NIH3T3 mouse fibroblasts, where PI3KC2β over-expression induced marked cell morphology changes in the cytoskeletal organization, including strong stress fibers formation, enlargement of the cell body, increased cell spreading and ruffles formation. I could demonstrate formation of a PI3KC2β/Dbl complex in mouse fibroblasts and human cancer cells. I showed that the N-terminal regulatory domain of PI3KC2β can interact with Dbl through its spectrin and pleckstrin holomogy (PH) domains revealing an interesting and novel

model of regulation. The association of PI3KC2β with Dbl however was not direct and did not depend either on the PI3K activity of the enzyme or on EGF and PDGF stimulation, suggesting a more complex mechanism of PI3KC2β-mediated Dbl activation and implication of additional interaction partners. Moreover, an interaction of PI3KC2β isolated from A-431 epidermoid carcinoma cells with oncogenic form of Dbl was found, indicating a potentially important role of the kinase in Dbl-dependent tumorigenesis.

In order to gain further insights into the PI3KC2β mechanism of regulation and function in cellular responses I have investigated for the first time the role of tyrosine phosphorylation of PI3KC2β in human cancer cells. Among four newly identified tyrosine phosphorylation sites three residues (Y68, Y127, Y228) were found in the N-terminal domain of the enzyme, whereas one (Y1541) was localized in the C-terminal extension. Simultaneous mutation of Y127 and Y228 in PI3KC2β wild-type increased HT-29 colon cancer cell proliferation, cell-cell junctions formation and activation of Akt/PKB pathway. Moreover, the cellular changes resulting from Y127 and Y228 mutation were similar to the effects of PI3KC2β kinase domain mutation when compared to PI3KC2β wild-type, indicating a possible role of tyrosine phosphorylation of the enzyme's N-teminal domain in the regulation of its kinase activity. Therefore, I propose that phosphorylation of Y127 and Y228 in the N-terminus of PI3KC2β wild-type negatively regulates cell proliferation, cell-cell junctions formation and activation of Akt/PKB pathway. Future work would be needed to elucidate the details of this regulatory mechanism and its function in tumor cells.

All together, these results unveil new aspects of PI3KC2β mechanisms of regulation, which have not been investigated before. They further provide new insights into the possible role of PI3KC2β in human cancer. Last but not least, they contribute to the growing understanding of the role of class II PI3Ks in physiological and pathological processes in human biology.

ZUSAMMENFASSUNG

Seit der Entdeckung der Proteinfamilie der PI3Ks (Phosphoinositid-3-Kinasen) in den achtziger Jahren des letzten Jahrhunderts, wurden die Isoformen der Klasse II der PI3Ks nicht ausgiebig untersucht und sie repräsentieren noch immer die am Wenigsten untersuchte Klasse der PI3Ks. Im Forschungsinteresse stand bislang hauptsächlich die Klasse I PI3K Enzyme, da diese eine gut etablierte Rolle in der Entwicklung verschiedener menschlicher Erkrankungen spielen, einschliesslich Diabetes und Krebs. Nichtsdestotrotz deuten eine zunehmende Anzahl von Studien neulich an, dass die Klasse II PI3Ks eine wichtige Rolle in verschiedenen physiologischen und pathologischen Prozessen spielen. Die wachsende Aufmerksamkeit für diese Klasse PI3Ks hat zwar zu einem besseren Verständnis über ihre zellulären Funktionen beigetragen, dennoch wurden die genauen Aktivierungsmechanismus und die Zielmoleküle dieser Enzyme bisher nicht vollständig enthüllt. Anhäufende Daten deuten darauf hin, dass die Klasse II PI3Ks sich von der Klasse I PI3K erstens in die intrazelluläre Lokalisation und zweitens in die Synthese anderer Phosphoinositide unterscheiden. Infolgedessen regulieren sie möglicherweise andere biologische Prozesse bzw. verschiedene Schritte im selben Prozess als die restliche PI3K Enzyme.

Die PI3KC2β-Isoform gehört zur Klasse II PI3Ks. Ihre zellulären Funktionen wurden bisher mit migrations- und überlebensfördernden Signalen, sowie mit der Zellproliferation assoziiert. Nach Ligandenstimulation wird das Enzym zu den aktivierten Rezeptortyrosinkinasen (RTKs) über die Adaptorproteine Grb2 oder Shc rekrutiert. An der Plasmamembran generiert PI3KC2β hauptsächlich PtdIns(3)P oder PtdIns(3,4)P_2 und bildet Multiproteinkomplexe, welche zur Aktivierung der Rho GTPasen und schlussendlich zur Regulierung des Akt/PKB Signalweges führen. Wie genau PI3KC2β zu den oben genannten Funktionen beiträgt, wurde noch nicht vollständig beschrieben. Allerdings scheint der Aktionsmechanismus mit der Multidomänenstruktur der Kinase zusammen zu hängen, welche stark von den Strukturen der Klasse I und III PI3Ks abweicht. Die charakteristischen Merkmale des Enzyms sind die fehlenden Assoziationen zu den regulatorischen Untereinheiten, welche von einer hohen molekularen Masse und verlängerten N- und C-terminalen Erweiterungen begleitet werden. Die N- und C-terminalen Erweiterungen spielen eine wichtige Rolle bei der Regulierung der katalytischen Aktivität von PI3KC2β. Der Zusammenhang zwischen den strukturellen Merkmalen von PI3KC2β und ihrem Wirkungsmechanismus sollte trotzdem noch eingehender untersucht werden.

In der vorliegenden Dissertation wurde der Regulationsmechanismus untersucht, welcher PI3KC2β mit der Aktivierung der Rho-Familie kleiner GTPasen via den Dbl-Guaninaustauschfaktor verknüpft. Diese Experimente wurden in NIH3T3 Mausfibroblastenzellen ausgeführt, in welchen die Überexpression von PI3KC2β zu

markanten Änderungen der Zellmorphologie in der Zytoskelettorganisation führte, einschliesslich starker Stressfasernbildung, Vergrösserung des Zellkörpers, erhöhte Zellausbreitung und Bildung von Membranausstülpungen. Die Bildung eines PI3KC2β/Dbl-Komplexes in Mausfibroblasten und menschlichen Krebszellen wurde bewiesen. Desweiteren wurde es gezeigt, dass die N-terminale, regulatorische Domäne von PI3KC2β mit Dbl durch dessen Spektrin- und Pleckstrinhomologie (PH)-Domänen interagieren können, was ein interessantes Regulierungsmodell einbringt. Die Komplexbildung zwischen PI3KC2β und Dbl war jedoch nicht direkt und weder von der PI3K-Aktivität des Enzyms, noch von der EGF- und PDGF-Stimulation abhängig. Dies deutet somit auf einen komplexeren Mechanismus der PI3KC2β-vermittelten Aktivierung von Dbl hin und impliziert die Anwesenheit von zusätzlichen Interaktionspartnern. Zudem wurde eine Interaktion zwischen PI3KC2β, isoliert aus A-431 Epidermoidkarzinomzellen, und der onkogenen Form von Dbl gefunden, was eine potentiell wichtige Rolle der Kinase in der Dbl-abhängigen Tumorentstehung hinweist.

Um weitere Einblicke in den Regulationsmechanismus und die Funktion der zellulären Reaktionen von PI3KC2β zu gewinnen, wurde zum ersten Mal die Rolle der Tyrosinphosphorylierung in der aus menschlichen Krebszellen isolierten PI3KC2β-Isoform untersucht. Von den vier neu identifizierten Tyrosinphosphorylierungsstellen wurden drei (Y68, Y127, Y228) in der N-terminalen Domäne und eine (Y1541) in der C-terminalen Erweiterung des Enzyms lokalisiert. Die gleichzeitige Mutation von Y127 und Y224 in PI3KC2β-Wildtyp erhöhte die Proliferation, die Bildung von Zell-Zellverbindungen sowie die Aktivierung des Akt/PKB-Signalweges in HT-29 Darmkrebszellen. Im Vergleich zum PI3KC2β-Wildtyp waren die Veränderungen, die aus Y127 und Y228 Mutationen resultierten, zudem ähnlich zu den Effekten, die aus Mutationen in der PI3KC2β-Kinasedomäne entstanden. Dies deutet auf eine mögliche Rolle der Tyrosinphosphorylierung der N-terminalen Enzymdomäne in der Regulierung der Kinaseaktivität hin. Dadurch kann man schliessen, dass die Phosphorylierung von Y127 und Y228 im N-Terminus des PI3KC2β-Wildtyps die Zellproliferation, die Bildung von Zell-Zellverbindungen und die Aktivierung des Akt/PBK-Signalweges negativ reguliert. Zwangsläufig ist weitere Arbeit nötig, um die Details dieses regulatorischen Mechanismus und dessen Funktion in Tumorzellen aufzuklären.

Zusammenfassend liefern die Ergebnisse der vorliegenden Dissertation neue Erkenntnisse über den Regulationsmechanismus von PI3KC2β, welche bisher noch nicht untersucht wurden. Sie bieten zusätzlich neue Einsichten in die mögliche Rolle von PI3KC2β in menschlichen Krebserkrankungen. Nicht zuletzt tragen sie zum wachsenden Verständnis der Rolle der Klasse II PI3Ks in physiologischen und pathologischen Prozessen in der Humanbiologie bei.

1. INTRODUCTION

1.1. Role of the Kinome and Tyrosine Phosphorylation in Cell Signaling

The understanding of signaling pathways in human cells and translation of this knowledge to improved human disease treatments is one of the most challenging tasks faced by the biologists and clinicians nowadays. Many aspects of signal transduction biology and biochemistry have been recognized and combined into a small number of unified principles, which help to interpret the system's complexity. However, still much remains to be learned in this area of research.

Intracellular signaling cascades are initiated by the ligand stimulation of cell surface receptors and are finalized in the nucleus with an induction of respective genes expression. The phosphorylation and dephosphorylation of proteins and lipid substrates play a fundamental role in signal transduction and are involved in almost all cellular processes such as proliferation, differentiation, adhesion, survival, cell cycle progression, metabolic homeostasis and transcriptional activation [1, 2]. The rapid and reversible nature of phosphorylation provides precise control of protein activity, cellular localization, stability, conformation and interaction with other proteins. Tyrosine phosphorylation is mediated by the 90 tyrosine kinases of the 518 protein kinases comprising the human kinome [1]. 58 of the 90 human tyrosine kinases are receptor tyrosine kinases (RTKs), while the remaining are classified as non-receptor tyrosine kinases [2]. In the last group, the Src non-receptor tyrosine kinase was the first tyrosine kinase identified [3]. Protein tyrosine phosphatases (PTP) act simultanously to tyrosine kinases antagonizing their activity by removing phosphate from phospho-tyrosines [4]. There are around 81 active PTPs identified with the ability to dephosphorylate phospho-tyrosine. Only the tightly controlled collaboration of protein kinases and phosphatases ensures the maintenance of cell homeostasis. Any perturbations in this perfectly balanced system may lead to human diseases progression, in particular cancer, which is characterized by aberrant signal processing. The role of tyrosine phosphorylation seems to be more diverse than it was originally assumed [2]. Most of the proteins become phosphorylated at different sites with different kinetics. Thus, phosphorylation of the same protein may be differently regulated at distinct sites leading to the integration of variety of upstream and downstream signals. Each of these phosphorylation sites can further play different functions [5], as it was already shown for many well-studied signaling proteins, for instance FAK or Src [6, 7]. It is therefore more

accurate to study site-specific phosphorylation rather than phosphorylation of the whole protein to gain a more precise knowledge about protein regulation.

During the last decade mass spectrometry (MS) has become a main method of choice for the *in vivo* investigation of phosphoproteome dynamics. Hypothesis-free mass spectrometry-based analysis of *in vivo* phosphorylation in a number of different cell lines and tissues under various conditions revealed thousands of simultaneously occuring tyrosine phosphorylation events in multiple proteins [5, 8-10]. Among all these proteins there are kinases and phosphatases, transcriptional and cytoskeletal regulators, ubiquitin ligases, GEF/GAPs and RNA-binding proteins. Many of the tyrosine phosphorylation sites have been already recognized and some were even functionally characterized, but there are still plenty left, which await identification and functional description [5].

1.2. Receptor Tyrosine Kinases

In the group of 58 known human receptor tyrosine kinases (RTKs) we can distinguish 20 subfamilies among which the most studied are the families of epidermal growth factor receptor (EGFR/ErbB), insulin receptor (InsR/IGF1R), platelet-derived growth factor receptor (PDGFR), vascular entothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), angiopoietin receptor (Tie) and ephrin receptor (Eph) [11]. All RTKs share the same molecular architecture that includes a ligand-binding extracellular domain, a single transmembrane helix, and a cytoplasmic protein tyrosine kinase domain that catalyses phosphate transfer from ATP to tyrosine residues. Additionally, carboxy (C-) terminal and juxtamembrane regulatory regions can be distinguished. RTKs play a key regulatory role in the cells. The structure, the mechanism of RTKs activation and components of the signaling pathways, which they trigger are evolutionary conserved from worms to humans [11]. The vast majority of RTKs consist of a single polypeptide chain while members of the insulin-receptor family are disulphide-linked heterodimers [12]. Regardless of the monomeric or dimeric form of the inactive receptor, the ligand binding is required to stabilize individual receptor molecules into an active hetero-dimers or oligomers. Ligand-mediated dimerization is therefore essential to promote RTKs activation [11]. However, it is not sufficient enough to induce activation of the intracellular kinase domain [13]. Due to dimerization, two kinase domains are brought together close enough to activate each other through *trans*-phosphorylation in the activation loop [11]. This mechanism plays a crucial regulatory role in the activation of most RTKs. However, an allosteric activation of the tyrosine kinase domain without phosphorylation of the activation loop is also possible. The EGFR represents the best example of such mechanism. The tyrosine kinase domains of two EGFR molecules form an

asymetric head-to-tail dimer, which is stabilized by the juxtamembrane segment [11, 14, 15]. Binding of one kinase domain called “Activator” to its partner domain denoted as “Receiver” results in conformational changes in the later one, which in turn adopts an active state [15]. Subsequent *trans*-autophosphorylation events in the juxtamembrane region and the C-terminal tail play an important role in terms of full RTK activation and downstream signal transduction. Respective tyrosine sites are phosphorylated in a precise order, which significantly increases receptor tyrosine kinase domain catalytic activity and creates the phoshotyrosine-based docking sites that recruit downstream signaling molecules [11]. In some RTKs (FGFR) an additional phase of autophosphorylation occurs, which maximally stimulates the tyrosine kinase domain for phosphorylation of downstream targets [16].

1.2.1. Phosphotyrosine Binding Motifs in Signal Transduction

Autophosphorylation of tyrosine residues in the cytosolic tail of RTKs is crucial for the recruitment and activation of a variety of signaling proteins, which recognize and specifically bind to the phosphorylated tyrosines via Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains [11, 17]. SH2- and PTB-domain-containing proteins function as molecular intergators of cell signalling pathways from the exterior to the interior of the cell recruiting diverse signaling molecules to the activated RTKs. Their binding specificity is directed respectively by the carboxyterminal or amino-terminal sequences that flank the phosphotyrosine residues on the receptor [18]. SH2 domains are divided into three classes: phospholipase C-γ1 (PLC-γ1)-like, Src-like and Grb2-like depending on the structural features of the binding surface [19]. Among SH2-domain-containing molecules we can distinguish intracellular, non-catalytic adaptor proteins such as Grb2 (growth factor receptor-bound protein 2), SHC, Crk and Nck. Moreover, enzymes with phospholipase (PLC-γ), tyrosine kinase (Src family of proteins), phosphatase (SHP-2) and ubiquitin ligase (c-Cbl) activity are included to that group [18]. The functional diversity of SH2-domain-containing proteins reflects the complexity of signaling pathways, which can be triggered downstream of activated RTKs. Ras/MAPK (mitogen-activated protein kinase) and PI3K (Phosphatidylinositol 3-Kinase)/Akt pathways are just two examples.

Many adaptors or proteins, which function as molecular scaffolds in signaling pathways can be found in the group of PTB-domain-containing molecules [18]. SH2-domain-containing docking protein SHC and insulin receptor substrate-1 (IRS-1) were the first identified members in this family [20]. PTB domains recognize NPXpY amino-acid sequences and were found in numerous signaling molecules [18]. Similarly to the SH2 motifs, they mediate

the activation of various signaling pathways such as the Ras/MAPK pathway, insulin receptor, Tek/Tie2, EGF and ErbB2 receptors downstream pathways.

1.2.2. Adaptor and Docking Proteins

Adaptor proteins can selectively recognize activated receptors with their SH2 domains, but simultanously they recruit cytoplasmic effectors with their Src-homology-3 (SH3) motifs, which bind to proline and arginine-rich sequences [21, 22]. Two SH3 domains of Grb2 associate with the Ras guanine-nucleotide exchange factor (GEF) son-of-sevenless (Sos), what results in formation of GTP-bound Ras and subsequent activation of the MAPK pathway [23]. Due to combined binding properties, simple adaptors control complex cellular behaviour by interactions with multiple partners in response to stimuli from the extra- and intra-cellular environemnt. Grb2 participates in the activation of many signaling pathways. Comprehensive quantitative and time-dependent studies of changes in protein interactions with Grb2 revelaed 108 Grb2-associated proteins including lipid or protein kinases, phosphatases, GTPase guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), adaptor and scaffolding proteins [22]. Many of them are involved in growth factor-specific signaling networks, which control distinct cellular functions like metabolism or cytoskeletal architecture. Some of the proteins like IRS4, p85 and p110α/p110β seem to be part of a core Grb2 signaling machinery. They showed increased association with Grb2 upon cell stimulation with the majority of growth factors, while Grb2 interactions with other proteins (EGFR, FRS2 and GAB1) were more growth-factor specific [22]. SH2- or PTB-domain-containing proteins can also associate to activated receptors indirectly through docking proteins, which become phosphorylated by the receptor, which they interact with [11, 24]. Docking proteins (FRS2, IRS1, Gab1) have the ability to associate with the plasma membrane through the membrane targeting of the N-terminal region (either by myristoylation or pleckstrin homology (PH) domain). Due to presence of whole array of tyrosine phosphorylation sites they serve as a binding platform for SH2- and PTB-domain-containing signaling molecules [11, 24].

With multiple tyrosine phosphorylation sites in all receptors and a wide range of proteins interacting with them, activated RTKs can transmit the signal from the exterior to the interior of the cell and stimulate diverse signaling cascades. Between these signaling pathways crosstalks often occur, which place RTKs in the position of key regulatory nodes in the complex and dynamic signaling networks [11].

1.3. Phosphatidylinositol 3-Kinases

Phosphatidylinositol 3-Kinases (PI3Ks) are additional key players in the intracellular signaling pathways, which makes membrane-to-cytosol communication even more complex [25]. They belong to the family of evolutionary conserved lipid kinases, which operate downstream of activated RTKs and G protein-coupled receptors (GPCRs) [25]. Thus, PI3Ks function at the level of the plasma membrane, where they phosphorylate phosphoinositides (PtdIns) at the 3-OH group of the inositol ring. As a result, these lipid kinases give rise to the formation of second messengers such as PtdIns(3)P (PtdIns 3-phosphate), PtdIns(3,4)P_2 (PtdIns 3,4-bisphosphate) and PtdIns(3,4,5)P_3 (PtdIns 3,4,5-trisphosphate) [26], which become targets for PH (pleckstrin homology), PX (Phox) and FYVE zinc finger-binding modules [27, 28]. Thus, PtdIns(3,4,5)P_3 controls the localization and function of numerous effector proteins, which after recruitment to the plasma membrane get activated. As a result, they promote various local cellular responses, such as assembly of signaling complexes, initiation of protein kinase cascades and actin polymerization [29, 30]. The Akt/PKB Ser/Thr kinase plays a central role in the PI3K pathway. It directly binds to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 via its PH domain and becomes phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at Thr^{308} [31, 32]. However, to acquire full catalytic activity Akt must be phosphorylated at Ser^{473} by the mammalian target of rapamacin complex 2 (mTORC2) [33]. Upon activation, Akt phosphorylates a wide range of target proteins, which affect important cellular processes [25, 29, 30]. Through the regulation of the tuberous sclerosis complex 1 and 2 (TSC1 and TSC2) Akt controls autophagy, cell cycle, as well as cell growth. Control of the cell growth is based on the modulation of the protein translation machinery through the mammalian target of rapamycin complex 1 (mTORC1) and its downstream targets, p70S6 kinase and 4E-binding protein 1 (4EBP1) [34]. Akt-mediated phosphorylation of murine double minute 2 protein (MDM2) increases MDM2 activity and in turn promotes degradation of the p53 tumor suppressor protein, which regulates apoptosis, cell cycle arrest and DNA-repair mechanisms [35]. Apoptosis-related substrates of Akt include also transcription factors from the forkhead (FOXO) family [29, 36], the proapoptotic protein BAD [35, 37] and the nuclear factor-kappa B (NF-κB) transcription factor [35]. Their phosphorylations lead to the prevention of apoptosis and promotion of cell survival through distinct molecular mechanisms. By inhibition of glycogen synthase kinase 3 (GSK3) Akt controls cell cycle and glucose metabolism in the insulin signaling pathway [29, 38]. An overview of the PI3K signaling pathway is given in Fig. 1-1.

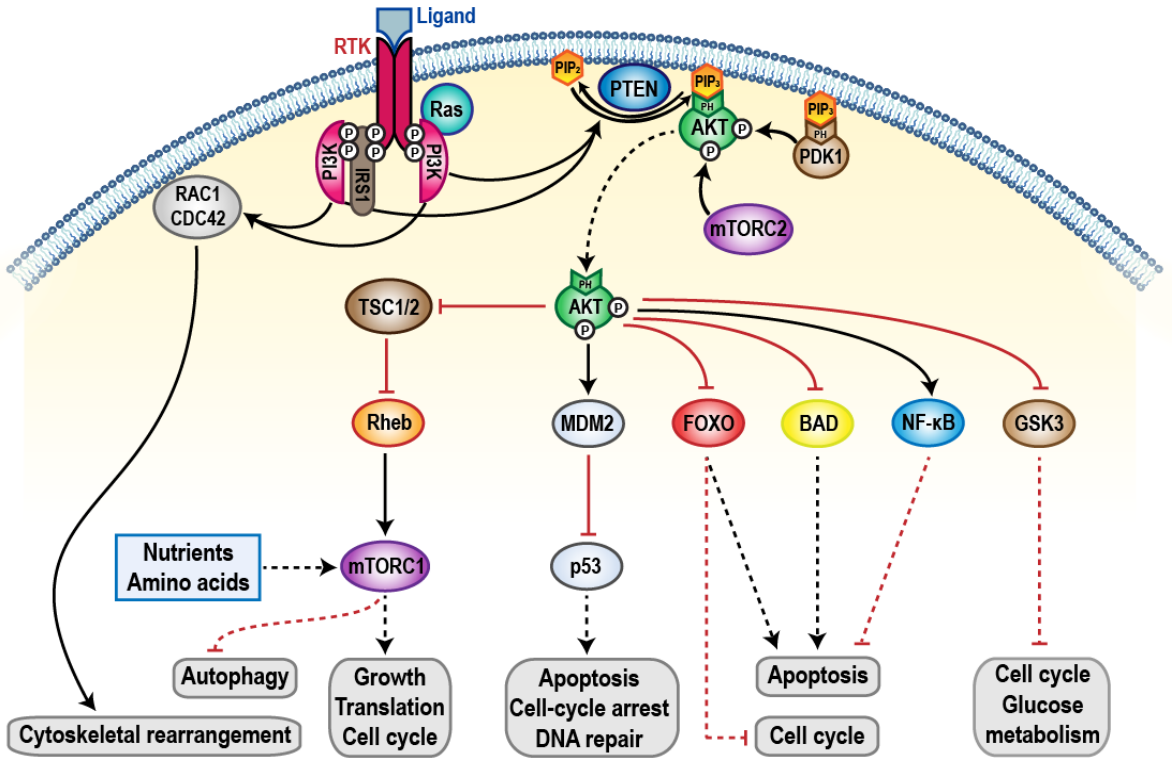

Figure 1-1. PI3K signaling pathway. PI3Ks are recruited to the plasma membrane by activated RTKs. They bind to phosphorylated receptors and generate PtdIns(3,4,5)P_3, which in turn become a targets for multiple PH-domain containing proteins including Ser/Thr kinases Akt and PDK1. PDK1 phosphorylates Akt at Thr^{308}, but to be fully activated Akt requires mTORC2 phosphorylation of Ser^{473}. Fully active Akt phosphorylates a number of downstream effector molecules leading to their activation or inhibition and thus resulting in cell growth, survival and proliferation through various molecular mechanisms. PI3Ks can also control cytoskeletal rearrangements through RAC1/CDC42, in an Akt-independent manner. Abbreviations: RTKs, receptor tyrosine kinases; PH, pleckstrin homology domain; PDK1, 3-phosphoinositide-dependent kinase 1; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamacin complex 2; FOXO, forkhead box, class O proteins; BAD, BCL-2 antagonist of cell death; Rheb, RAS homologue enriched in brain. Adapted from Chalhoub and Baker, 2009 [30].

PI3K/Akt signaling is under the tight control of two different types of phosphatases, which balance PI3Ks activities by dephosphorylation of (PtdIns(3,4,5)P_3) [29]. The Src-homology 2 (SH2)-containing phosphatase (SHIP) dephosphorylates the 5-position of the inositol ring producing PtdIns(3,4)P_2, whereas the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates the 3-position of PtdIns(3,4,5)P_3 producing PtdIns(4,5)P_2 [29, 39]. As specified above, PtdIns(3,4)P_2 can play a role of second messenger independently of PtdIns(3,4,5)P_3 By generation of PtdIns(3,4)P_2 SHIP proteins do not terminate PI3K signaling, but convert one signal to another. PTEN, on the other hand, completely inactivates the PI3K signal not giving a chance for induction of further signaling cascades [39]. Thus, SHIP and PTEN phosphatases play an important role in antagonizing the PI3K pathway and maintaining cell signaling homeostasis. Loss of SHIP2 leads to

increased sensitivity to insulin [40], whereas loss of PTEN function has been often found in many types of advanced human cancers [30]. In addition to PTEN and SHIP, myotubularins were found to antagonize PI3Ks activity by dephosphorylation of PtdIns(3)P [41]. Myotubularins recently have received a lot of interest in the context of actin cytoskeleton remodeling and membrane trafficking [42].

In the last years, the physiological functions of PI3Ks have been widely studied in cell line–based systems and model organisms. They appear to be extremly important in diverse cellular responses including cell proliferation, differentiation, chemotaxis, survival, intracellular trafficking and glucose homeostasis. Furthermore, the enzymes are involved in the embryonic development and immunity. Therefore, aberrant PI3K activity is associated with a wide range of human diseases including cancer, diabetes, heart and respiratory disorders, as well as inflammation [43]. Somatic mutations in *PIK3CA* and *PIK3R1*, encoding respectively p110α and p85, promote the activation of the PI3K/Akt pathway and are frequently found in human cancers [44]. Similarly, *PIK3CA* gene amplifications significantly contribute to cancer progression. Most of the mutations in p110α are located in two “hot spot” regions in exon 9 and 20, which encode the helical and kinase domain, respectively. Mutations in the helical domain disrupt the inhibitory effect of p85 on the p110α catalytic subunit, whereas mutations in the kinase domain increase the p110α enzymatic activity [44]. The second leading mutation in human cancer occurs in the *PTEN* gene. It leads to phosphatase inactivation and thus hyper-activation of PI3K/Akt pathway, which enhance tumor cells survival and proliferation. Usually, the expression of PTEN is altered through loss of heterozygoty (LOH), point mutations, homozygous deletion or epigenetic silencing via promoter methylation [45]. Somatic mutations or amplifications of Akt were also frequently found in cancer. The most common mutation (E17K) affects the lipid binding site (PH domain) causing constant association to the plasma membrane, even in the absence of PtdIns(3,4,5)P_3 [46].

1.3.1. Classification of the PI3K Family Members

It took around 10 years of research since the first PI3Ks were discovered until they became classified in the 1990s [25]. The PI3K family consists of 8 catalytic subunits, which emerged from the PCR-based cloning studies and biochemical purification strategies. They were used to identify sequence homologies between lipid kinases and to characterize PI3K enzymatic properties, respectively [25]. All members of the family show structure similarities. They possess a core sequence consisting of C2, helical and catalytic kinase domains [47]. On the other hand, PI3Ks differ in their substrate preferences *in vitro* and their association

with regulatory subunits. Based on these criteria PI3Ks have been divided into three functional classes: class I (p110α, p110β, p110δ and p110γ), class II (PI3KC2α, PI3KC2β and PI3KC2γ) and a single class III (Vps34) enzyme [43, 48]. Class I is further divided into class I_A and I_B, depending on the receptor type, which regulates their activity. All PI3Ks are conserved throughout evolution. Class I and class II are found in multicellular eukaryotes (invertebrates and vertebrates such as *C.elegans, D.melanogaster,* mouse and human), whereas simple unicellular eukaryotes (e.g. yeasts) and all plant species investigated so far possess only a sole class III PI3K [43, 48]. Many members of the family are ubiquitously expressed in humans, while others are confined to specific cells and tissues [43, 48]. For a detailed classification, PI3Ks substrate specificity, upstream regulators, cellular function, tissue distribution, as well as PI3Ks domain structure see Table 1-1 and Fig. 1-2. In addition to the three PI3K classes, a class IV of PI3K-related proteins (PIKKs) exists in all eukaryotes. It includes mTOR (mammalian target of rapamacin), ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia mutated related) and DNA-PK (DNA-dependent protein kinase) Ser/Thr kinases, whose sequences suggest a common evolution from lipid kinases and in turn similar regulation [48-50]. Most of the PIKKs function at the genomic level sensing the presence of DNA damage and maintaining its integrity [49, 50]. mTOR is an exception, because it is involved in cell growth, protein synthesis and autophagy signaling.

Class I PI3K was the first to be identified and cloned, and thus the best understood until now. Enzymes of this class can phosphorylate *in vitro* PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 giving rise to PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, respectively. *In vivo* PtdIns(4,5)P_2 is the main substrate and respectively PtdIns(3,4,5)P_3 is the main product [43, 51]. They are cytosolic proteins, which are recruited to the membrane upon ligand stumulation, where they bind to activated receptors or adaptor proteins [48]. Depending on the regulatory partners and mechanism of activation (including the receptor type they bind), class I PI3Ks is divided into class I_A and class I_B. Class I_A PI3Ks comprises a group of catalytic (p110α, p110β, p110δ) and regulatory (p85α, p85β, p55γ, p55α, p50α) subunits, which in human are encoded by three genes: *PIK3CA, PIK3CB, PIK3CD* and *PIK3R1, PIK3R2, PIK3R3*, respectively. Due to alternative splicing the three genes encoding the regulatory subunits can give rise to five different isoforms.

Table 1-1. Classification of PI3K family members based on their substrate specificity, regulation and cellular functions.

Classification of PI3Ks						
	Isoforms	**Gene name**	**Substrate specificity**	**Regulator**	**Cellular function**	**Expression**
Class I_A	**Regulatory** p85α, p55α, p50α p85 β p55γ **Catalytic** p110α p110β p110δ	 *PIK3R1* *PIK3R2* *PIK3R3* *PIK3CA* *PIK3CB* *PIK3CD*	 PtdIns PtdIns(4)P PtdIns(4,5)P_2	 • RTKs • GPCRs (p110β) • Ras	 • proliferation • metabolism • migration • survival • immunity (p110δ)	 Ubiquitous p110δ mainly in leukocytes
Class I_B	**Regulatory** p101 p84/p87 **Catalytic** p110γ	 *PIK3R5* *PIK3R6* *PIK3CG*	 PtdIns PtdIns(4)P PtdIns(4,5)P_2	 • GPCRs • Ras	 • inflammation • platelet aggregation • immunity	 Mainly in leukocytes
Class II	**Catalytic** PI3KC2α, PI3KC2β PI3KC2γ	 *PIK3C2A* *PIK3C2B* *PIK3C2G*	 PtdIns PtdIns(4)P	 • RTKs • Cytokine receptors • Integrins • GPCRs (LPA)	 • vesicular transport • cell migration • chemotaxis	 Broad expression, but not ubiquitous PI3KC2γ mainly in liver
Class III	**Regulatory** Vps15/p150 **Catalytic** Vps34	 *PIK3R4* *PIK3C3*	 PtdIns	 • GPCRs • amino acids • glucose	 • autophagy • vesicular transport	 Ubiquitous

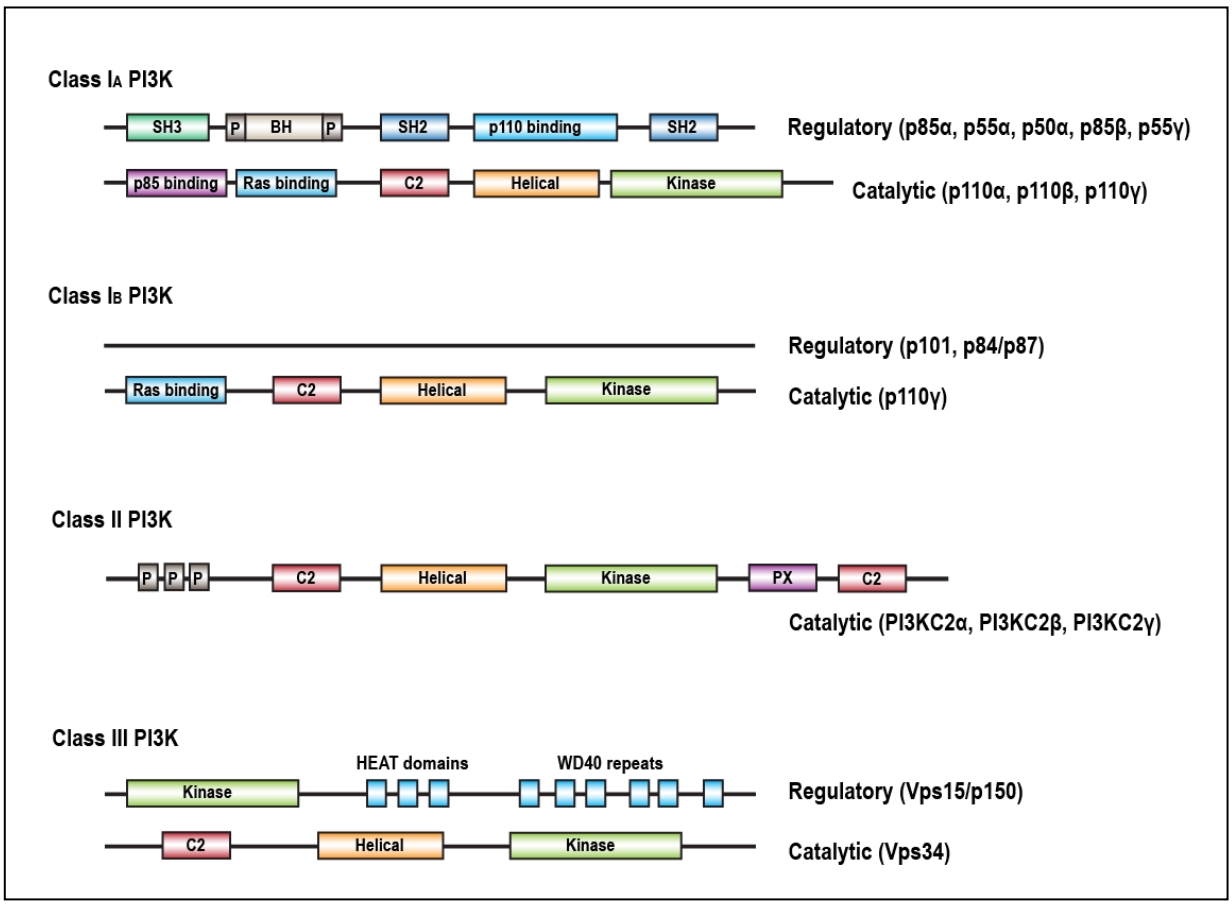

Figure 1-2. Domain structures of PI3Ks. With the exception of class II enzymes, all PI3Ks consist of regulatory and catalytic subunits. PI3K catalytic subunits include a C2 domain and a PI3K core structure containing a kinase and helical domain. Class I_A regulatory subunits (p85α, p55α, p50α, p85β, p55γ) consist of the main p110-binding domain and two SH2 domains, which bind to tyrosine-phosphorylated residues at the activated RTKs. p85α and p85β additionally contain a SH3 domain, which can attach to proline-rich regions of other proteins, and BH domain flanked by two proline-rich regions (P). The shorter splicing variants of p85α lack the N-terminal BH- and SH3 domain. Beside domains common for all PI3Ks catalytic subunits, class I_A catalytic isoforms contain N-terminus p85-binding domain responsible for the interaction with the regulatory subunit, and Ras-binding domain. The catalytic p101γ isoform of class I_B has the same domain structure as class I_A apart from the lack of p85 binding domain. Class I_B regulatory isoforms, p101 and p87 do not have any homology to other proteins and this far, none of their domains have been identified. Class II PI3Ks comprises of three high molecular mass catalytic isoforms (PI3KC2α, PI3KC2β, PI3KC2γ) and are characterized by an additional C2 and PX domain within the C terminus and a proline-rich region (P) in the N-terminus, which differs between the three isoforms. The catalytic subunit of class III PI3Ks, Vps34, consists of the PI3K core structure accompanied by N-terminal C2 domain. The regulatory isoform, Vps15 (also known as p150) contain a kinase domain, which is thought to be inactive, and several WD40 repeats and HEAT domains. Abbreviations: RTKs, receptor tyrosine kinases; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain; BH, BCR homology domain; PX, Phox domain. Adapted from Blajecka *et* al. 2011 (Appendix).

The catalytic isoforms, together with five regulatory subunits can form heterodimers with each other and can potentially give rise to fifteen distinct p85-110 combinations [47]. They become activated through direct interactions with broad variety of RTKs, or in some cases (such as p110β) also by interaction with GPCRs [47, 51]. Binding with the receptor releases the basal inhibition of p110 by p85 and brings the p85-p110 complex in the close proximity to

its substrate PtdIns(4,5)P_2 at the plasma membrane [52, 53]. The p85 N-terminal binding domain of p110 isoforms is used for interactions with regulatory subunits, while the Ras-binding domain mediates enzyme activation through the Ras GTPase [47, 54]. The class I_A regulatory isoforms consist of a p110-binding domain that is flanked by two SH2 domains, which bind specifically to phosphorylated tyrosine residues on autophosphorylated receptors or on target docking proteins (such as IRS1) [17]. In addition to that, N-terminal extension of p85α and p85β subunits consisting of SH3 and proline-rich motifs can be found, which mediate binding to other signaling molcules. The breakpoint cluster region-homology (BH) domain plays a role of GTP-ase activating protein (GAP) for small GTPases [47, 54]. Class I_B is represented by a single member p110γ encoded by the *PIK3CG* gene. p110γ has been identified only in mammals. It lacks a p85-binding domain, but has three regulatory subunits: p101, p84 and $p87^{PIKAP}$, which have no identifiable domains and do not show homology to any other proteins. Due to these structural differences, the class I_B enzyme is not activated by RTKs, like class I_A enzymes. Its activation is linked exclusively to GPCRs [47, 54]. p110γ is translocated to the membrane (Gβγ subunit of GPCR) from the cytosol through the interaction with its regulatory subunit p101, which is required for p110γ activation [55]. Class I_B PI3K can be also activated via Ras-mediated mechanisms by allosteric modulation [56].

The importance of class I PI3K in physiological processes has been widely investigated. Animal model studies have greatly contributed to our understanding of their function. As indicated by murine knock-outs of the *PIK3CA* and *PIK3CB* genes, which appeared to be lethal at the embryonic stage, p110α and p110β are essential for basic cellular functions in the organisms [57, 58]. p110α seems to be important for cell survival whereas p110β is essential for cell proliferation. Both isoforms also play an important role in insulin signaling in a kinase-dependent and independent fashion, respectively [51, 59, 60]. On the other hand, the class I_A p110δ and class I_B p110γ are key players in inflammation. Mice deficient in p110δ were viable, but had severely impaired T and B cell function [61]. p110γ has been shown to be involved in the regulation of thymocyte development, T cell activation, neutrophil migration and the oxidative burst [62, 63].

Class III PI3K in human consists of a sole 100 kDa catalytic subunit and a single 150 kDa regulatory subunit [64]. It was first identified in a *Saccharomyces cerevisiae* screen for genes required for vacuolar protein sorting and vacuole seggregation [65]. Originally isolated as a mutant in yeasts, it was called vacuolar protein-sorting defective 34 (Vps34) and it is encoded in human by the *PIK3C3* gene. It phosphorylates only PtdIns generating PtdIns(3)P and forms a heterodimer with the regulatory subunit, termed as Vps15/p150. Vps15 is myristoylated, which results in the attachment of the Vps34-Vps15 complex to intracellular membranes. Moreover, Vps15 acts as Ser/Thr kinase and its activity is essential for Vps34 function [47]. Vps34 acts downstream of GPCRs forming multiprotein complexes and its

activation can be regulated by nutrients (amino acids or glucose). In general, it is involved in intracellular trafficking like autophagy, endocytosis and phagocytosis. It was also shown to regulate the mTOR/S6K1 pathway in response to amino acids avaibility suggesting a possible involvement in cell growth control [66]. The class III Vps34 is present in all eukaryotes [47].

1.4. Class II PI3Ks

Class II PI3Ks were identified in 1990s based on the PCR-based search for the lipid kinase domain sequence homologies with other PI3Ks known at that time (p110α, p110β, p110γ, Vps34). The functional or physiological context of class II PI3Ks discovery was missing. For that reason they are the least studied and understood among all PI3K family members. There are still plenty of questions concerning their regulation and function, which remain unanswered. For instance, what is the class II PI3Ks definitive substrate and product *in vivo* upon different ligands stimulation? Are the class II PI3Ks redundant with other PI3Ks or do they rather act cooperatively? What is the significance of their unique domain structure for their precise mechanism of action? Fortunately, the interest in solving all these issues is now growing, which hopefully will lead to an increase in our knowledge about this interesting class of PI3K enzymes [67].

1.4.1. Identification and Expression

In mammals there are three class II isoforms identified: PI3KC2α, PI3KC2β and PI3KC2γ encoded respectively by *PIK3C2A, PIK3C2B* and *PIK3C2G* genes [47, 48, 67]. A single representative member of class II PI3Ks is present in multicellular invertebrate organisms such as *C.elegans* and *D.melanogaster*. In worms it is exemplified by F39B1.1 hypothetical protein (GenBank accession no. NM_078128), whereas in the fruit fly by PI3K_68D/Cpk. In vertebrate genomes exemplified by human or mouse we can find all three representatives of class II enzymes [48]. The first discovery of class II PI3Ks began with the identification of PI3K_68D/Cpk in *D. melanogaster* [68, 69]. Soon after, murine analogue Cpk-m and p170 were cloned from the mouse brain and liver cDNA libraries and from the insulin-sensitive mouse 3T3-L1 adipocytes, respectively [69, 70]. These isoforms displayed lipid kinase activity *in vitro* towards PtdIns and PtdIns(4)P and were characterized by the presence of Ca^{2+}-independent C2 domain at the carboxy-terminus. The different substrate specificity and structural features compared to other known PI3Ks led scientists to believe that distinct PI3K isoforms exist, which may play divergent roles in cell biology. These

assumptions were confirmed when the first human class II PI3K isoforms were identified. HsC2-PI3K (named later PI3KC2β) was isolated from a cDNA library of the breast tumor cell line MCF7 [71], and further from the U937 (human leukemic monocyte lymphoma) cDNA library [72]. The HsC2-PI3K gene was localized on chromosome 1 (1q32) and the mRNA tissue distribution appeared to be ubiquitous with the highest expression level in thymus and placenta [71]. PI3KC2β used as substrates PtdIns and PtdIns(4)P in the presence of Mg^{2+} and showed sensitivity to low concentration of wortmannin similarly to class I_A PI3Ks. However, it was slightly more resistant to the LY294002 inhibitor than p110α/p85 subunits [72]. In contrast to PI3KC2β, PI3KC2α was rather insensitive to wortmannin and LY294002 at concentrations, which inhibited the catalytic activity of other PI3K isoforms [73]. PI3KC2α (localized at chromosome 11p15.5-p14) was cloned from U937 cells and was found to be widely expressesd in tissues. The highest expression levels were observed in heart, placenta and ovary [73]. It could phosphorylate PtdIns and PtdIns(4)P *in vitro* and to some extend PtdIns(4,5)P_2 in the presence of phospatidylserine. The whole picture of class II PI3K members was complemented when PI3KC2γ was identified in mouse, rat and human [74-76]. The human *PIK3C2G* gene was mapped to chromosome 12p12 and displayed an expression pattern restricted to the liver, breast and prostate [76]. In contrast to PI3KC2α and PI3KC2β, high expression of PI3KC2γ was confined to the liver also in mouse and rat, suggesting a tissue-specific role of this isoform [74, 75].

1.4.2. Substrates Preferences *In vitro* and *In vivo*

Since the first studies concerning class II PI3Ks substrate specificity were published it is generally accepted that the main substrates *in vitro* of this class of enzymes comprise of PtdIns and PtdIns(4)P. However, some indications exist that PtdIns is the class II PI3Ks preferential substrate [67]. As mentioned above, PI3KC2β phosphorylated PtdIns and PtdIns(4)P in the presence of Mg^{2+}, but in fact the kinase activity towards PtdIns(4)P was only 10% of that oriented towards PtdIns [72]. Addition of phosphatidylserine, phosphatidylcholine, or phosphatidylethanolamine significantly increased the enzyme activity toward PtdIns in comparison to PtdIns(4)P. Moreover, only PtdIns but not PtdIns(4)P were phosphorylated by PI3KC2β and PI3KC2α, when Ca^{2+} was added to the *in vitro* kinase assay as a source of divalent cation [77]. The hypothesis that PtdIns(3)P is the major product of class II PI3Ks was also supported by several *in vivo* studies. It was demonstrated in the rat skeletal muscle L6 cells and 3T3-L1 adipocytes that insulin induce PtdIns(3)P synthesis in the TC10 guanine exchange factor–mediated mechanism [78]. This PtdIns(3)P production appearded to be resistant to high concentrations of wortmannin and LY294002, which

suggested PI3KC2α's involvement in this process. This was indeed proven in later studies, where PI3KC2α downregulation completely abolished insulin-dependent generation of PtdIns(3)P, while it did not block formation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, [79]. Thus, PtdIns(3)P seemed to be the exclusive PI3KC2α product upon insulin stmulation. Moreover, in further research on neuroexocytosis, PtdIns(3)P was found as PI3KC2α main product [80, 81]. Nevertheless, in recent studies in MIN6 pancreatic β-cells it was observed that in response to insulin release upon glucose stimulation PI3KC2α generates PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3, which become second messengers for selective PKBα/Akt1 activation [82]. PI3KC2α signaling towards PKB/Akt involves insulin receptor B type (IR-B) and leads to stimulation of B-cell glucokinase (*βGK*) gene transcription and to an increase in the activity of Akt substrate AS160 [82]. This results indicate that although PI3KC2α preferentialy uses PtdIns as a substrate *in vivo*, the generation of PtdIns(3,4)P_2 cannot be completely excluded. PtdIns(3)P also seems to be the main *in vivo* product of PI3KC2β. It was shown to produce PtdIns(3)P at the plasma membrane in response to lysophosphatidic acid (LPA) stimulation of HeLa and ovarian cancer cell line SKOV-3 [83]. siRNA downregulation of PI3KC2β inhibited the LPA-dependent GFP-2XFYVE plasma membrane translocation. GFP-2XFYVE is able to bind only to PtdIns(3)P. Therefore, an expression of GFP-2XFYVE in HEK293 cells stably transfected with PI3KC2β completely abolished PI3KC2β-dependent formation of lamellipodia and filopodia due to the blockade of PtdIns(3)P signaling [84]. Moreover, synthesis of PtdIns(3)P was also demonstrated by the nuclear fraction of PI3KC2β, whose activation occurs at the G_2/M phase of the cell cycle [85]. All these results taken together confirm the hypothesis that PtdIns(3)P is the main product of PI3KC2β activity *in vivo,* at least upon the LPA stimulation. However, more data concerning stimulation with other growth factors are needed to fully support this conclusion. No information is available so far about *in vitro* and *in vivo* products of PI3KC2γ.

1.4.3. Structural Characteristics

Class II enzymes share the same core sequence (C2, helical and catalytic domains) with other PI3Ks, but some of their structural features differ considerably [47, 48, 67]. The major contrast is that class II proteins are monomers of high molecular mass and they do not associate with any regulatory subunits. Lack of the regulatory subunit-binding domain is compensated by the N- and C-terminal extensions, which possess other domains enabling interactions with proteins or with the plasma membrane via phospholipids. The C-terminal extension is highly conserved between the three isoforms and it contains a Phox homology (PX) domain, known to bind phosphoinositides [86], and an additional C2 domain, whose

function is not fully understood. However, the C2 C-terminal region is the most distinctive feature of class II PI3K's structure. Generally, C2 domains have membrane-targeting properties and single or multiple copies are present in a variety of intracellular proteins involoved in signal transduction or membrane trafficking [87]. It can bind to a remarkable number of ligands and substrates, such as Ca^{2+}, phospholipids, inositol polyphosphates, as well as cytosolic proteins. Initially, it was identified in the protein kinase C (PKC) as a Ca^{2+}-dependent lipid binding module. However, soon it became clear that it can also act in a Ca^{2+}-independent fashion. It was unexpectedly observed that the C2 domain of PKCδ binds directly to phosphotyrosine peptides providing new possibilities for the C2 domain-dependent regulation of molecular processes [88]. The C2 C-terminal domain of the class II PI3Ks binds phospholipids independently of Ca^{2+} [72, 87, 89]. Moreover, in the case of PI3KC2α and PI3KC2β it contains a nuclear localisation signal (NLS), which directs the proteins to nuclear speckles or the nuclear matrix, respectively [90, 91]. In contrast to the C-terminal extension, class II PI3K's N-terminal extensions differ between the isoforms. For instance, PI3KC2α's N-terminal sequence contains a clathrin-binding motif [92], as well as PI3KC2β, which displays four potential clathrin-binding fragments [93]. However, only *D.melanogaster* PI3K_68D/Cpk and human PI3KC2β possess type II polyproline stretches (PPLPPR), which can serve as targets for SH3-domain-containing proteins such as Drk or Grb2 adaptor proteins, respectively [68, 69, 71, 94, 95]. The N-terminal sequence of PI3KC2α differs much from the previously described PI3K_68D/Cpk and murine proteins [73]. The most important feature is that it does not contain the polyproline region II, although numerous proline residues are present [73]. PI3KC2γ on the other hand, does not show homology with any of the known proteins and its function remains unknown [74]. Based on the sequence similarity to class I PI3Ks, a Ras-binding domain was distinguished in the class II isoforms [67]. Nonetheless, no interaction with GTP- or GDP-bound form of Ras could be observed, as for the PI3KC2β [72]. To see the structure of class II PI3Ks refer to Fig. 1-2.

1.4.4. Mechanisms of Activation

Class II PI3Ks, in particular PI3KC2α and PI3KC2β, are activated via cellular stimulation with various types of ligands, which is followed by recruitment of the isoforms to the plasma membrane. Among PI3KC2α stimuli we can distinguish hormones such as insulin [79, 96], chemokines like monocyte chemotactic protein 1 (MCP1) [97], cytokines such as leptin and TNFα [98] and some of the growth factors, for instance epidermal growth factor (EGF) [77]. Analogously, PI3KC2β is mostly activated by growth factors like EGF, platelet-derived growth factor (PDGF) and stem cell factor (SCF) [77, 99]. However, it was also

shown to become activated upon lysophosphatidic acid (LPA) stimulation, which is a phospholipid derivative stimulating G protein-coupled receptors (GPCRs) [83]. Interestingly, both PI3KC2α and PI3KC2β are activated by insulin, although in contrast to PI3KC2α, PI3KC2β's biological role in the IR pathway was not studied [79, 82, 100]. Both of the isoforms are downstream targets of integrins. A decrease in PI3KC2α activation was observed in migrating vascular smooth muscle cells (VSMC) after blockade of the $\alpha_v\beta_3$ integrin [101], whereas an increase in PI3KC2β activity was detected after $\alpha_{IIb}\beta_3$ integrin and fibrinogen–dependent human platelets aggregation [102]. All these data taken together suggest that class II PI3KC2α and PI3KC2β are activated downstream of RTKs [77, 79, 96, 99] and GPCRs [83, 97]. Unfortunately, no data are available concerning PI3KC2γ activation.

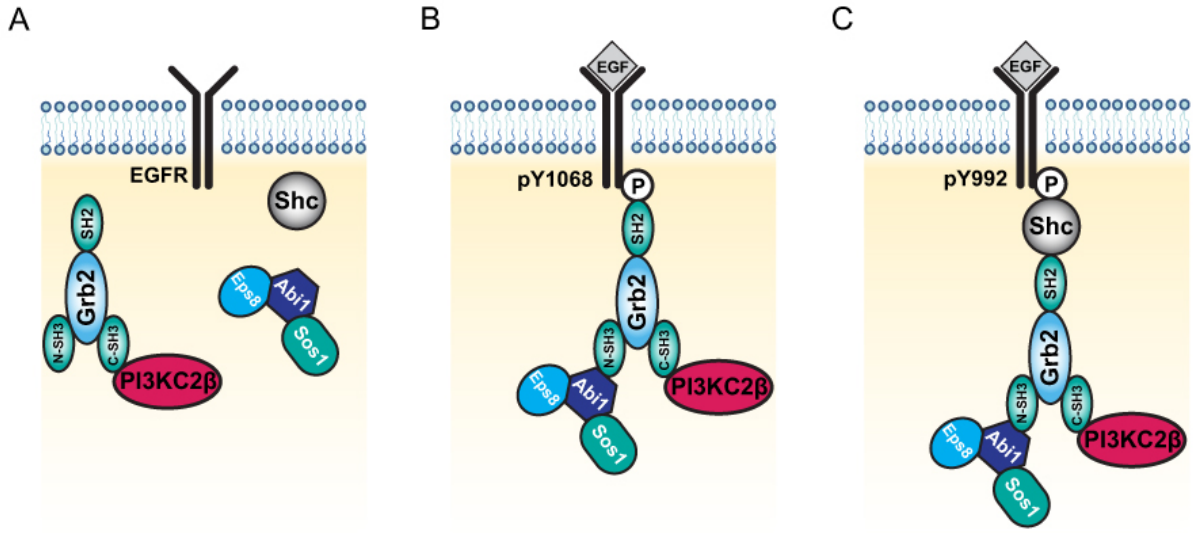

Figure 1-3. PI3KC2β protein-protein interactions and translocation to the membrane. In quiescent cells PI3KC2β is complexed with Grb2 in A-431 cells through the Grb2 SH3 C-terminal domain (A). Upon EGF stimulation, the multiprotein complex of PI3KC2β-Grb2-Abi1-Eps8-Sos1 is recruited to the activated EGFR either directly on Y1068 through association with Grb2 (B) or indirectly on Y992 through interaction with Shc (C). Formation of this macromolecular complex promotes PI3KC2β lipid kinase activity and induces signal transduction to Rac GTPase, which in turn leads to cell-cell adherens junction assembly and F-actin polymerization. Adapted from Katso *et* al. 2006 [103].

Plasma membrane translocation plays an important role in the mechanism of PI3Ks activation where association of the kinases with activated growth factor receptors takes place. Binding of the kinases to membrane receptors is often mediated by interactions with adaptors or scaffolding molecules, GTP-binding proteins and guanine nucleotide exchange factors (GEFs). For instance, PI3KC2β was shown to bind multiple RTKs, which was followed by enzyme activation. Among these receptors we can find c-Kit, c-Met and IGF-IR in

small cell lung cancer cells (SCLC), as well as PDGFR and EGFR in HEK293 and A-431 cancer cells [77, 99]. Detailed studies of PI3KC2β interaction with the EGFR in A-431 cells revealed an indirect association through Grb2 and Shc adaptor proteins [94]. Moreover, an Eps8/Abi/Sos1 ternary complex, playing a role of guanine exchange factor for Rac, was involved in the interaction in a lipid kinase activity-independent manner [103] (Fig. 1-3). Increased PI3KC2β activity was reported upon EGF stimulation in Eps8, Abi1 and Shc immunoprecipitates. Beside that, PI3KC2β association with the modular scaffolding protein interesctin (ITSN) increased the enzyme activity measured by PI3K assays and Akt activation under both basal and EGF-stimulated conditions in neuroblastoma cancer cells [104]. All these results, taken together suggest an important function for protein-protein interactions in class II PI3K activation at the plasma membrane.

Furthermore, the domain structure of class II PI3Ks is also crucial for the enzyme activation. Since class II kinases do not have the possibility to bind regulatory subunits such as class I PI3Ks, they developed extended N- and C-termini carrying additional protein domains, which could possibly play this role [67]. Examples of such regulation can be found in each isoform of class II PI3K. For instance, deletion of PI3KC2α's clathrin-binding N-terminal domain extremely enhanced the enzyme's kinase activity toward PtdIns when compared to PI3KC2α wild-type in the absence of clathrin. This result suggests that the N-terminal extension plays a role of negative regulator of PI3KC2α activity. When clathrin was added to the *in vitro* kinase assay in the presence of full-length PI3KC2α, a positive regulation was observed [92]. The role of clathrin-binding module and binding of clathrin itself in the control of PI3K enzymatic activity seems to be meaningful, which is supported by the fact that clathrin-binding motifs in the PI3KC2β N-terminus have been linked to its increased activation [93]. Furthermore, the proline-rich regions of the PI3KC2β N-terminal extension have been shown to be important for the kinase activity. Deletion of the first proline-rich region rendered the enzyme catalytically inactive, and blocked PI3KC2β interaction with the activated EGFR and Grb2. Again, this emphasizes importance of the associated proteins for the regulatory role of the class II PI3K. Further deletions that removes the second and third proline-rich motifs increased PI3KC2β activity [93]. Analogously, removal of the unique C2 C-terminal domain from PI3KC2β resulted in elevated PI3K activity suggesting that it functions as a negative regulator of the catalytic domain [72]. In PI3KC2γ, both the N-terminal and C-terminal extensions were shown to be essential for the enzyme activity [74].

There is not much information about post-translational modifications, which could contribute to class II PI3Ks activation. Tyrosine phosphorylation of PI3KC2α upon insulin stimulation was observed in CHO-IR cells, although this has not been further investigated [96]. Similarly, PI3KC2β tyrosine phosphorylation upon SCF, HGF, FGF2 and insulin stimulation was detected in SCLC cell line H-209 [99]. Both isoforms were also

phosphorylated on tyrosine in A-431 and HEK293 cells under basal and EGF-stimulated conditions, although much more of PI3KC2α and PI3KC2β was immunoprecipitated by anti-phosphotyrosine antibody from cell lysates after EGF treatment [77]. Interestingly, tyrosine phosphorylation of PI3KC2β isolated from nuclei and nuclear envelopes of ATRA-differentiated HL-60 cells was observed, which further correlated with an increase in the activity of PI3KC2β and accumulation of nuclear PtdIns(3)P. These results suggest that tyrosine phosphorylation might be essential for enzyme activation. However, this hypothesis needs further investigations.

Translocation from the cytosol to the plasma membrane seems to be crucial for the activation of class II PI3Ks [67]. Since both PI3KC2α and PI3KC2β contain nuclear localization signal (NLS) in their C-terminal C2 domains, it cannot be excluded that the kinases may become activated after relocation to the nucleus [90, 91]. However, localization of the kinases in different subnuclear compartments (nuclear speckles and matrix, respectively) raises the possibility that PI3KC2α and PI3KC2β might play distinct intranuclear functions. Increase in PtdIns(3)P levels as a result of PI3KC2β activation was found in the membrane-depleted nuclei during compensatory liver growth. Interestingly, proteolysis mediated by calpains may contribute to this activation, but only in the absence of cell membrane and cytosolic fractions, which possibly contain factors, which inhibit proteolysis [105]. It is likely that the enzyme activation in the nuclear matrix occurs as a result of the C2 C-terminal domain cleavage, which was shown to play a negative regulatory role for PI3KC2β activity [72, 106]. A similar mechanism of calpain-mediated PI3KC2β activation was described in the nuclei and nuclear envelopes of HL-60 leukemia cells during the G_2/M transition of the cell cycle [85]. Moreover, a significant increase in the activity of the enzyme was detected in the nuclei and nuclear envelopes isolated from all-*trans*-retinoic acid (ATRA)-differentiated HL-60 cells [107]. Stimulation of HEK293 cells expressing recombinant PI3KC2β with EGF increased the enzyme level and activity in the nuclei and revealed PI3KC2β co-localization with lamin A/C in the nuclear matrix [91].

A novel mechanism of regulation of PI3KC2β activity was described recently [108]. An interaction of PI3KC2β with TRIM27 (tripartite-motif-containing protein) was obsereved, which resulted in increased ubiquitination at the Lys^{48} of the lipid kinase. However, polyubiquitination did not induce protein degradation, but inhibited its catalytic activity [108].

1.4.5. Cellular and Physiological Functions of Class II PI3Ks

Due to the *in vivo* generation of phospholipid products distinct from class I PI3Ks, class II PI3Ks most likely activate different downstream signaling pathways. Whether they control the same or different cellular processes is not clear. However there are reports in the literature, which confirm that class I and class II PI3Ks can act together to regulate the same cellular responses [67]. With respect to the class III PI3Ks, class II enzymes share the same substrate *in vivo* (PtdIns), but distinct cellullar compartmentalization of both classes of enzymes exludes functional redundancy. Next to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, PtdIns(3)P can also act as dynamic intracellular second messenger, which could be involved in many signaling pathways [109]. Since class II PI3KC2α and PI3KC2β produce the same lipid products, are both ubiquitously expressed, bind to clathrin with their N-terminus, and are both recruited to the activated EGFR, it is very likely that they play complementary roles in some biological functions. Indeed, both enzymes were shown to control cell growth and survival pathways. Down-regulation of PI3KC2α induced apoptotic changes in Chinese hamster ovary (CHO-IR) cells, HeLa cells and hepatocellular carcinoma cell line [110-112]. Reduced cell proliferation and reduced anchorage-independent growth were additionally observed [112]. On the contrary, over-expression of PI3KC2α enhanced mesenchymal stem cell survival under hypoxic conditions in the infarcted myocardium [113]. Increased proliferation and resistance to anoikis were detected in A-431 epidermoid carcinoma cells over-expressing wild-type PI3KC2β in comparision to parental cells. Interestingly, both of these responses were not mediated by Akt activation [103]. Additionally, over-expression of PI3KC2β in intersectin-silenced mouse N1E-115 and human IMR5 neuroblastoma cells, rescued the survival of N1E-115 cells during differentiation and restored anchorage-independent growth of IMR5 cells [104, 114].

Other physiological functions of PI3KC2α and PI3KC2β do not seem to overlap. PI3KC2α's biological role is mostly associated with insulin signaling and glucose transport [79, 82, 115], endocytosis [92, 116], neurosecretory granule release and VSMC contraction [80, 81, 117-119], whereas PI3KC2β controls mainly cell migration processes [83, 84, 103], cell-cycle progression [85] and K^+ channel activation [108, 120]. The biological functions of PI3KC2γ are not known to date. The only report describing a possible role of this isoform in chemotaxis and abnormal homing of leukemic cells has been published recently [121]. The authors suggest that down-regulation of PI3KC2γ by p185(Bcr-Abl) may suppress SDF1α-induced chemotaxis, leading to abnormal homing of leukemic cells [121].

Since PI3KC2β is the main subject of this thesis, its cellular and physiological functions will be described in more detail than for other class II PI3Ks. As mentioned above,

cell migration is the major process, which is regulated by this isoform. PI3KC2β over-expression in HEK293 cells decreased the expression of the $\alpha_{IV}\beta_1$ integrin subunits and cell adhesion [84]. These changes correlated with increased cell migration supported by lamellipodia and filopodia formation, which was dependent on PtdIns(3)P and activation of the Cdc42 RhoGTPase [84]. In other studies, a LPA-dependent pool of PtdIns(3)P was generated through PI3KC2β activation and was involved in the cell migration of ovarian and cervical cancer cell lines [83]. Furthermore, over-expression of the kinase in A-431 epidermoid carcinoma cells revealed Rac-mediated increase in membrane rufflng and migration speed of the cells when compared to A-431 parental cells either in the presence or absence of EGF [103]. On the other hand, transfection of kinase-dead PI3KC2β into the A-431 cell line reduced cell migration speed. Accumulation of F-actin and E-cadherin at cell-cell junctions, as well as lamellipodia formation was also observed in the cells over-expressing the kinase, while the kinase-dead transfected cells displayed perinuclear E-cadherin assembly [103].

Despite the unquestionable role of PI3KC2β in the cell migration and adhesion processes, its involvement in T-cell activation was recently discovered [120]. The enzyme can be activated downstream of the T-cell receptor (TCR). This further stimulates the activation of the K^+ channel KCa3.1, which maintains a negative membrane potential necessary for accurate Ca^{2+} entry into the cells. siRNA down-regulation of PI3KC2β considerably suppressed KCa3.1 activity, which was due to decreased levels of PtdIns(3)P [120]. The detection of increased PI3KC2β activity in the nuclei and nuclear envelopes of HL-60 cells progressing into the G_2/M-phase indicates a possible enzyme involvement in the cell cycle control [85]. However, its specific functions in this proccess were not examined to date.

Mouse knock-out models have contributed to the knowledge of class II PI3K's functional relevance. PI3KC2γ is the least studied member of the family. Therefore, knock-out or knock-in *in vivo* transgenic models for this isoform are missing. A recently published murine knock-out of PI3KC2α revealed that the PI3K is essential for normal postnatal development, support of podocyte function and maintenance of normal renal homeostasis [122]. PI3KC2α-depleted mice were considerably smaller than the wild-type animals. Approximately 30% of *PI3KC2α* -/- individuals died by 6 months of age in comparison to 5% of wild-type animals. Severe symptoms of renal failure and a wide range of kidney lesions were detected in *PI3KC2α*-depleted mice [122]. In the case of murine PI3KC2β knock-out, a potential role in epidermal differentiation was investigated. However, no phenotype was observed in either suprabasal or basal epidermal layers. PI3KC2β-deleted mice were viable and fertile and were characterized by the lack of any abnormalities in epidermal growth, differentiation, barrier function and wound healing [123].

1.4.6. Involvement of Class II PI3Ks in Cancer

There are only few reports describing the role of PI3K class II isoform PI3KC2α in cancer. Its importance for cell growth and survival was shown in hepatocellular carcinoma [112]. Silencing of PI3KC2α significantly reduced cell proliferation and colony formation and increased caspase-3 activity, suggesting the PI3K involvement in apoptotic pathway. An interesting hypothesis explaining PI3KC2α role in cancer cell growth was suggested recently by Schepeler and colleagues [124]. They revealed that in colorectal cancer cells PI3KC2α is suppressed at the translational level by *miR-30e-3p*. In connection with this hypothesis they proposed a model where PI3KC2α becomes up-regulated upon down-regulation of the microRNA, which is possibly followed by an increase in colorectal cancer cell growth. In this model, the Wnt pathway may also play an important role, but this interpretation requires further verification. Moreover, PI3KC2α was also detected in acini and ducts of pancreatic ductal adenocarcinoma and in healthy tissue. However, higher expression of the kinase was observed in acini with high cellular atypia and in dysplastic ducts [125]. Among other genes, *PI3KC2A* was found to be highly expressed in the side population cells (SP) within the human breast cancer MCF7 cell line, which is known to be enriched in cancer stem-like cells, and display greater tumorigenicity *in vivo* in comparison to non-SP MCF cells [126]. Like in all other aspects, also in terms of malignant transformation, there is little information available so far about class II PI3KC2γ. There is only one publication, which demonstrates PI3KC2γ repression in the p185 (Bcr-Abl)–transformed murine Ba/F3 leukemia cell line. The authors suggested that p185 (Bcr-Abl)-mediated inhibition of PI3KC2γ contributes to the attenuation of chemotaxis and abnormal homing of leukemic cells [121]. Interestingly, very recent studies in non-small cell lung cancer (NSCLC) identified intronic mutations in *PI3KC2A* and *PI3KC2G* genes, however mutations of *PI3KC2G* were more common [127].

PI3KC2β is quite extensively studied in the context of cancer. It may be either over-expressed and amplified or under-expressed and deleted, although the consequences of these genetic alterations are not always described. Over-expression of the kinase in colonic epithelial cells led to oncogenic transformation represented by increased soft-agar colony formation and formation of transformed foci on cell monolayers [128]. Increased expression of the PI3KC2β enzyme was detected in different acute myeloid leukemia (AML) cell lines depending on molecular and cytogenetic abnormalities, which they bear [129]. Moreover, over-expression of the *PI3KC2B* gene was detected in therapy-related AML, a neoplastic disorder arising from a multipotential hematopoietic stem cell [130]. Decreased expression of *PI3KC2B* in acute lymphoblastic leukemia (ALL) possessing a chromosomal translocation involving the mixed-lineage leukemia gene (*MLL*, *HRX*, *ALL1*) was observed in comparison to convenvtional acute lymphoblastic leukemia (ALL) [131]. Deletion of *PI3KC2B* in ALL was

correlated with a decrease in MSH2 protein level (DNA mismatch repair enzyme), which consequently led to impaired DNA mismatch repair capacity in human leukemia cells [132]. Beside leukemias, *PI3KC2B* alterations were also examined in invasive intraductal papillary mucinous neoplasm (IPMNs) of the pancreas, where over-expression of the gene was detected [133]. On the other hand, *PI3KC2B* DNA copy number gain was identified in ovarian cancer, which was higher than the copy number gain for other PI3K family members. However, a significant up-regulation of mRNA in ovarian cancer versus normal ovary was observed only for *PIK3R3* (p55γ subunit) [134].

Over-expression or amplification of genes often correlates with drug resistance in cancer cells, which need to promote additional signalig pathways to overcome drug-induced stress. Amplification of *PI3KC2B* (1q32) was identified in 6 glioblastomas of 103 analysed. However, only in 4 of these cases amplification was accompanied by mRNA over-expression [135]. Gain at 1q32.1 (*PI3KC2B/MDM4*) was also reported by Nobusawa *et.* al. [136]. Further analysis of 1q32 chromosome sections revealed a single region of amplification encompassing *MDM4, GAC1, PI3KC2β and PEPP3* genes, with *MDM4* being the main amplification target always associated with over-expression [137]. Amplification of *PI3KC2B/MDM4* has been reported in glioblastoma multiform (GBM) [138], where *PI3KC2B* and *IGF1* expression significantly correlated with cellular resistance towards erlotinib, which is an EGFR tyrosine kinase inhibitor [139]. Down-regulation of PI3KC2β with siRNA in ERα (oestrogen receptor α)-positive MCF7 breast cancer cells and their further treatment with tamoxifen, an inhibitor of ERα signaling, sensitized the cells to the drug, suggesting that the kinase may be involved in tamoxifen resistance in breast cancer cells [140]. The involvement of PI3KC2β in mechanisms of drug resistance was recently supported by studies in oesophageal squamous carcinoma (ESCC) cells after the enzyme over-expression or silencing. PI3KC2β over-expression in Eca109 cells caused a 4-fold reduction in sensitivity to cisplatin and increased protection against apoptosis, whereas the kinase silencing rescued Eca109 cells' sensitivity to the drug and made them more susceptible to programmed cell death. Originally, the Eca109 parental cell line does not express PI3KC2β and due to that it was much more sensitive to cisplatin than other ESCC cell lines displaying the protein expression [141]. On the other hand, knock-down of *PI3KC2B* in human leukemia cells CEM increased the resistance of these cells against specific chemotherapeutics, which was associated with reduction of MSH2 and thus suppression of DNA mismatch repair capacity [132].

Over-expression of class II PI3KC2β in subsets of patient tumor samples and cell lines from AML, glioma, medulloblastoma, neuroblastoma, and small cell lung cancer not only reduced sensitivity to some chemotherapeutic agents, but also influenced some of the cellular responses. Pharmacological inhibition or siRNA-induced suppression of the enzyme

activity impaired cell proliferation and survival of AML, brain tumors and neuroendocrine malignancies [129]. In small cell lung cancer (SCLC) PI3KC2β contributed to stem cell factor (SCF)-stimulated Akt/PKB activation and cell growth, which was completely abolished when SCLC cells were transfected with a kinase inactive PI3KC2β construct [99]. Moreover, the PI3KC2β pathway turned out to be essential for neuroblastoma tumorigenesis by rescuing anchorage-independent growth of intersectin-silenced neuroblastoma cells [114]. Anchorage-independent growth or in other words protection of cancer cells against anoikis were shown to be regulated by over-expression of PI3KC2β in A-431 epidermoid carcinoma cells. Enhanced membrane ruffling and migration speed of these cells were also identified as a result of Rac-dependent mechanisms [103]. Cancer cell migration and related processes, such as cell invasion, protection against anoikis and cell adhesion are the most frequently observed cellular responses under the control of PI3KC2β [83, 129]. Recent results from 61 PI3KC2β-positive and –negative ESCC patients tissues revealed that there is a significant association between PI3KC2β protein levels and metastasis [141]. 68.2% PI3KC2β-positive cases showed metastasis compared to only 31.8% of PI3KC2β-negative cases. These results strongly suggest that PI3KC2β may play an important role in metastasis by regulation of cancer cell migration and invasion.

Interesting data concerning PI3KC2β mutations in cancers have started to emerge recently. Frequent somatic missense exonic mutations of *PI3KC2B* have been found in adenocarcinomas and squamous cell carcinomas of non-small cell lung cancer (NSCLC), making the kinase a promising druggable target for NSCLC therapy [127]. Moreover, single-nucleotide polymorphisms in the promoter region and in the first two introns of *PI3KC2B* were identified. SNPs were shown to be significantly associated with prostate cancer risk, especially for men diagnosed before age of 65 or for men with a family history of prostate cancer [142].

1.5. Cytoskeletal Rearrangements in Cancer Cell Migration and Adhesion

The ability of the cell to remodel its own cytoskeleton is an important feature, which is involved in the processes of cell migration and cell adhesion to other cells or to the substratum. These, on the other hand, are crucial for many physiological and pathological processes such as embryonic morphogenesis, wound healing, immune surveillance, tissue homeostasis, but also cancer, atherosclerosis, rheumatoid arthritis and many other disorders [143]. To metastasize to distinct organs and invade distal tissues giving rise to secondary tumors formation, cancer cells must be able to detach from the primary tumor mass, invade into the blood or lymphatic vessels, escape from the vessels and attach to the substratum in a new environment. For all these steps tumor cells need to constantly reorganize their cytoskeleton and change cell polarity. These processes require the activation of multiple signaling pathways, among which PI3Ks and RhoGTPases play a pivotal role [143, 144]. Intercellular adhesion is mediated by cadherins, a large family of transmembrane molecules, which require Ca^{2+} for their homophilic interactions mediated by the extracellular domains on the surface of the neighboring cells [145]. Clusters of E-cadherin homodimers at the cell-cell junctions can also bind to the actin cytoskeleton through cytoplasmic adaptor proteins. The assembly of E-cadherin complexes involves the activation of small GTPases, as well as recruitment of PI3Ks [145-147]. Loss of cellular adhesion in the epithelial structure is an early event in primary carcinogenesis and is linked to the onset of most solid tumors [148]. Upon loss of adherens junctions, primary tumor cells undergo epithelial-to-mesenchymal transition (EMT), which enable them to migrate into the circulatory or lymphatic system and to metastasize into distinct organs. Sometimes, aggregates of E-cadherin-positive cells get into the bloodstream, which helps the tumor cells survive mechanical and immunocytotoxic stresses [148].

1.5.1. Rho GTPases and Their Regulators

The Rho family of GTPases belongs to the superfamily of Ras-related small GTPases (~21 kDa) and consists of more than 20 members among which RhoA, Rac1 and Cdc42 are the best studied [149, 150]. They are highly conserved in evolution from lower eukaryotes to plants and mammals and are implicated in many basic cellular processes such as actin and microtubule cytoskeletal organization, cell cycle, cell migration and adhesion, vesicular trafficking, phagocytosis and transcriptional regulation of gene expression [150]. The expression and activity of Rho GTPases are frequently deregulated in cancer playing a major

role in tumor cell motility, invasion, metastasis and inflammation, as well as proliferation and survival [149, 151].

Rho GTPases act as molecular switches cycling between an active GTP-bound conformation and an inactive GDP-bound from [150]. Most of the Rho GTPases are modifed at their C-terminus by a prenyl moiety, which acts as a lipid anchor and localizes them to the membrane [152]. When small G proteins are active, they can interact with effectors to induce downstream signaling responses. The process of spatio-temporal activation and inactivation of Rho GTPases is tightly controlled by associating proteins termed guanine nucleotide exchange factors (GEFs), that catalyze the exchange from GDP to GTP, thereby contributing to turning on Rho GTPases downstream signaling [152]; GTPase activating proteins (GAPs) that terminate the signaling by inducing intrinsic GTP hydrolysis [152]; and guanine nucleotide dissociation inhibitors (GDIs), which inhibit GDP dissociation and whose role is to remove small G proteins from the membrane and solubilize them back to the cytosol [153] (Fig. 1-4). RhoA, Rac1 and Cdc42 are the most well characterized Rho GTPases, which are under the control of PI3Ks [144].

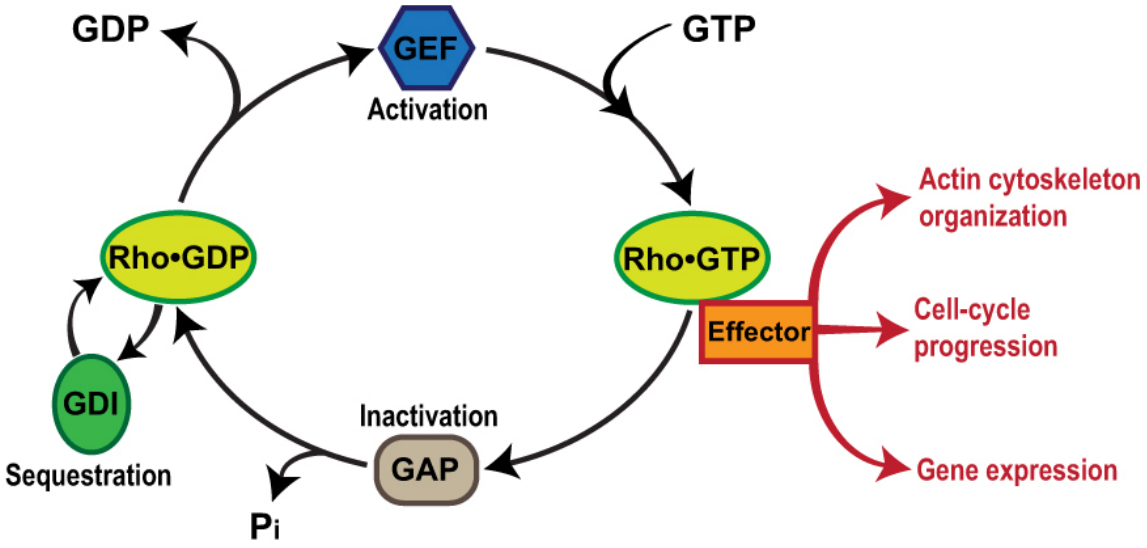

Figure 1-4. Regulation of the Rho GTPase activity. Rho GTPases are considered functional when they are bound to GTP, and not functional when they are GDP-bound. GEFs catalyze GDP to GTP exchange utilizing abundance of intracellular GTP, and regulate the activity of downstream effectors. These effectors are responsible for triggering different cellular responses such as actin cytoskeletal organization, cell-cycle progression or expression of various genes. GAPs stimulate the intrinsic hydrolytic activity of Rho GTPases promoting the GDP-bound inactive state and terminating signal transduction. GDIs inhibit GDP dissociation and sequester Rho GTPases in the cytosol, before they become targets for GEFs. Abbreviations: GEF, guanine exchange factor; GAP, GTPase activating proteins; GDI, guanine nucleotide dissociation inhibitors; P_i, inorganic phosphate.

Rac is responsible for the generation of lamellipodia or ruffles along the edge of the cell. RhoA regulates stress fibers formation, which are elongated actin bundles that promote cell attachment to the extracellular matrix by generation of focal adhesions. Cdc42 induces filopodia formation, thin spike-like membrane extensions responsible for the recognition of the extracellular environment [154].

The over-expression or increased activity of RhoGTPases, which was found in human cancer, led researchers to believe that GEFs and GAPs significantly contribute to tumor progression by promoting aberrant GTPase activation [151, 155]. Recently, mutation of proline 29 to serine in highly conserved switch I domain of Rac1 has been found in sun-exposed melanomas [156]. In the studied cohort, it was the third most frequent activating mutation after those of *BRAF* and *NRAS*, which was predominant in male patients when compared to females. This gender difference was unique for *RAC1*P29S and it was not found for mutations in *BRAF* or *NRAS.* Moreover, *RAC1*P29S alteration increased binding of RhoGTPase to downstream effectors and thus promoted melanocyte cell proliferation and migration [156]. There are around 85 GEFs and 80 GAPs identified in mammals [149, 150]. Several GEFs were mutated or aberrantly expressed in human cancer. One of the well known examples is the Rho-specific GEF Bcr, whose gene, as a result of the fusion with the Abl tyrosine kinase gene (fusion-gene known as Philadelphia chromosome), contributes to leukemia development [157]. The Rac-specific GEFs Tiam1 and Vav, which regulate Rac/Rho/Cdc42 are other examples, which were found deregulated in human cancer. The first one was identified as an invasion and metastasis-inducing gene in a murine T-lymphoma cell line, and the other one showed a wide range of effects on development and progression of human malignacies mostly due to its over-expression [155]. Considering their high oncogenic potential GEFs have been proposed as therapeutic targets for cancer [155].

GEFs catalyze GDP dissociation from small G proteins by modification of the nucleotide-binding site, which leads to a decrease in nucleotide affinity and its subsequent replacement with GTP [152]. The mechanism of GEFs activation strongly depends on their multi-domain structure. The minimal functional module consists of Dbl homology-Pleckstrin homology tandem domains (DH-PH), which display functional interdependence. They are respectively responsible for the intrinsic GEF catalytic activity toward RhoGTPases and intracellular distribution into the plasma membrane or into the cytoskeletal matrix, where the substrates of Dbl family proteins reside [158]. Beside the DH-PH motif, GEFs possess other protein domains, which are responsible for the specific cellular functions of the different family members. All these domains can bind to each other at the intramolecular way, but they can also serve as a binding platform for other proteins or lipid products. In this way GEFs become an integral part of large macromolecular complexes that are precisely coordinated for the highly dynamic spatio-temporal activation of Rho GTPases [158].

1.5.2. The Role of Class I PI3Ks in the Regulation of Rho GTPases in Cancer

PI3Ks collaborate with Rho GTPases in actin remodeling to establish cell polarity, definie the leading edge of the cell, regulate the cell migration, as well as protrusion and adhesion formation [144]. Numerous positive feedback loops and crosstalks exist between Rho GTPases and PI3Ks pathways, which integrate and amplify cell signaling. Rho GTPase may be activated in a PI3K- independent and –dependent fashion through GEFs such as Vav, Sos, Tiam, αPIX, p-Rex1 [159]. Upon stimulation of RTKs, GPCRs and extracellular matrix (ECM)-cell or cell-cell adhesion receptors (e.g. integrins, cadherins), GEFs may bind to PI3Ks lipid product PtdIns(3,4,5)P_3 [144].

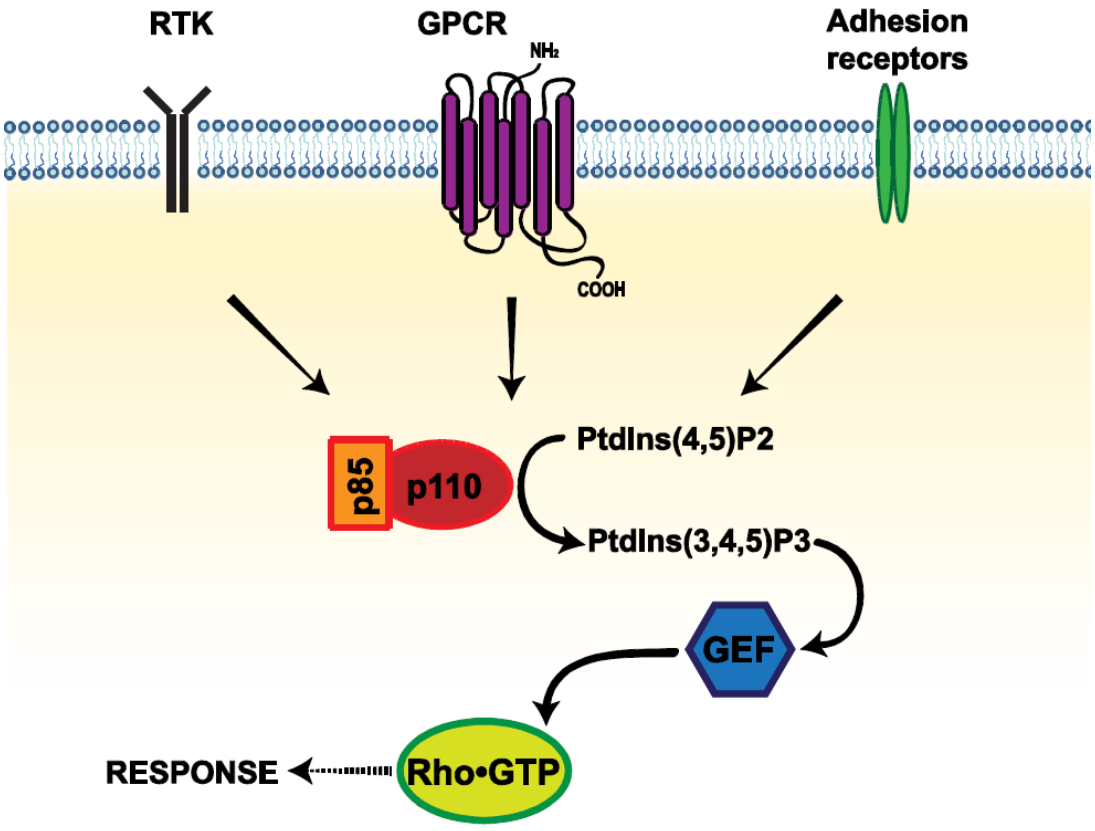

Figure 1-5. Class I PI3Ks role in Rho GTPases activation. Stimulation of RTK, GPCR and adhesion receptors activate class I PI3Ks, which generate PtdIns(3,4,5)P_3 second messengers. Phosphorylated lipids become targets for the Rho GEFs, which are recruited to the plasma membrane and promote activation of Rho GTPases residing at the membrane proximity. Activation of Rho GTPases causes induction of various cellular responses.

When GEFs are recruited to the membrane, they form multi-protein complexes with PI3K, RhoGTPases, as well as actin-binding proteins [160], which localize them to dynamic actin structures and ensures efficient RhoGTPase activation [160, 161]. p85 regulatory subunit of class I_A was shown to interact with Rac-specific GEF complex Eps8/Abi1/Sos1, in which Abi1 is responsible for the interaction, whereas Sos1 binds to PtdIns(3,4,5)P_3 and induces its GEF's activity towards Rac [161]. Inactivation of p110α, but not of p110β, impaired endothelial cells migration *in vitro* and *in vivo* through RhoA-dependent signaling during the angiogenesis [162]. In neutrophils of transgenic mice, which were deficient of class I_B p110γ, lack of generation of PtdIns(3,4,5)P_3 and significantly reduced motility upon GPCR stimulation were observed [163]. Further pharmacological inhibition of p110δ with IC87114 compound suppressed polarized morphology of neutrophils, fMLP-stimulated PtdIns(3,4,5)P_3 production and directional chemotaxis [164]. PI3K signaling inhibition with wortmannin or LY294002 has been extensively used to demonstrate their impact on cell polarity, migration and disruption of actin structures. Deregulation of PI3Ks in cancer influence RhoGTPases signaling leading to over-activation of the pathway. This, in turn, has been shown to contribute to most steps of cancer initiation and progression [151]. Class I PI3Ks role in the regulation of Rho GTPases is given on Fig. 1-5.

1.5.3. The Prototypic Dbl GEF and Its Oncogenic Counterpart

The *dbl* oncogene (MCF-2) was the first GEF identified based on DNA isolation from the human diffuse B-cell lymphoma (~66 kDa) and subsequently from nodular poorly differentiated lymphoma (NPDL-*dbl*) (~76 kDa) and the human mammary carcinoma cell line MCF-7 [165-167]. Further transfection of these DNAs into NIH3T3 cells induced cellular transformation uncovering Dbl's oncogenic potential. The *dbl* oncogene encodes a protein of 478 amino-acids that is produced as a result of loss of the first 497 amino-acids of proto-*dbl* (925 amino-acids, 115 kDa) located on chromosome X. Beside this modification, the genesis of all independently found onco-*dbl* forms involves acquisition of different fragments of genes from another human locus (chromosom 3) adding 50 amino-acids to the N terminus [166, 168, 169]. However, it is the loss of the 497 amino-acids and not the fusion with distal genes fragments, which is crucial for increased transforming activity of the *dbl* oncogene in NIH3T3 cells [168]. Over-expression of proto-*dbl* is sufficient to transform NIH3T3 cells and to produce tumors in nude mice. However, truncation of the N-terminal portion of the protein markedly enhances its transforming activity and tumorigenicity [168, 170]. Both proto- and onco-Dbl are cytoplasmic proteins, which function at the plasma membrane, but also associate wih the cytoskeletal matrix [171]. Both are mostly phosphorylated on serines,

although proto-Dbl is phosphorylated to lesser extent. The half-life of proto-Dbl is significantly shorter than the half-life of onco-Dbl, suggesting poor stability and rapid turnover [171]. All these differences may be related to distinct conformations of both proteins. The oncogenic Dbl conformation might be more prone to phosphorylation and confer greater stability either by increased protein-protein interactions or by enhanced resistance to proteases [171].

The expression of proto-*dbl* seems to be highly tissue-specific. Among the range of normal human tissues tested proto-*dbl* mRNA was detected only in fetal brain and adrenal glands, as well as adult testes and ovaries [170]. There are four splicing variants of the human proto-*dbl*, which display various GEF specificities and are differently expressed in brain, heart, kidney, spleen, liver, testis, placenta, stomach and peripheral blood [172]. There is approximately 75% cDNA and amino acids sequence homology between human DBL and its mouse counterpart MCF-2. Mouse *mcf-2* mRNA is expressed in brain, kidney, intestine and testis [173].

The GEF activity of onco-Dbl was demonstrated for the first time when it became clear that the amino acid sequence of human proto-Dbl between residues 498 and 738 shares significant similarity with cell-division-cycle protein Cdc24 present in *S. cerevisiae*, which was known to act on the GTP-binding protein Cdc42Sc [174]. Soon after, it was shown that oncogenic Dbl catalyzes the dissociation of GDP from human Cdc42 GTPase and thereby plays a role of guanine nucleotide exchange factor [175]. These findings were followed by additional reports, which showed the activity of onco-Dbl not only toward Cdc42, but also RhoA GTPase, whereas the Dbl GEF activity directed toward Rac1 GTP-binding protein was not detected [176]. Other studies demonstrated onco-Dbl-mediated dissociation of GDP from all three Rho GTPases, but for RhoA and Rac1 it was more rapid than for Cdc42 [177]. It seems then that oncogenic Dbl may act on all three Rho GTPases, although its activity torward RhoA and Cdc42 is more evident. On the other hand, proto-Dbl GEF activity is dependent on particular splice variants, which are expressed in different tissues and organs [172]. For instance, the variant 1 of Dbl, which is designated as prototype Dbl and is expressed only in the brain, showed the least activity directed toward RhoA, Rac1, Cdc42 when compared to the other three splice variants expressed in other organs. Variant 4 displayed high GEF activity torward Cdc42 and RhoA, whereas variant 3 exhibited weak activity toward Cdc42 and Rac1 in contrast to variant 1 [172]. Analogously to human variant 1, brain-specific mouse MCF-2 was also less active in the GDP-dissociation assay [173]. Studying GEF activity of particular Dbl forms in connection with their specific expression would certainly lead to interesting insights into Dbl function.

As in all GEF family members, the minimal functional DH-PH module can be found also in the proto-Dbl structure [178-180]. The spectrin domain of proto-Dbl located at the N-terminus regulates Dbl activity by indirect binding to PH-domain. This interaction blocks the

access to the catalytic DH domain and masks intracellular targeting function of the PH domain [178-180]. This indirect binding is mediated by chaperone heat shock cognate protein (Hsc70) complexed with chaperone Hsp90 and cochaperone CHIP, which is an E3 ubiquitin protein ligase [179, 180]. A schematic representation of proto-Dbl structure and regulation of its activity is shown on Fig. 1-6.

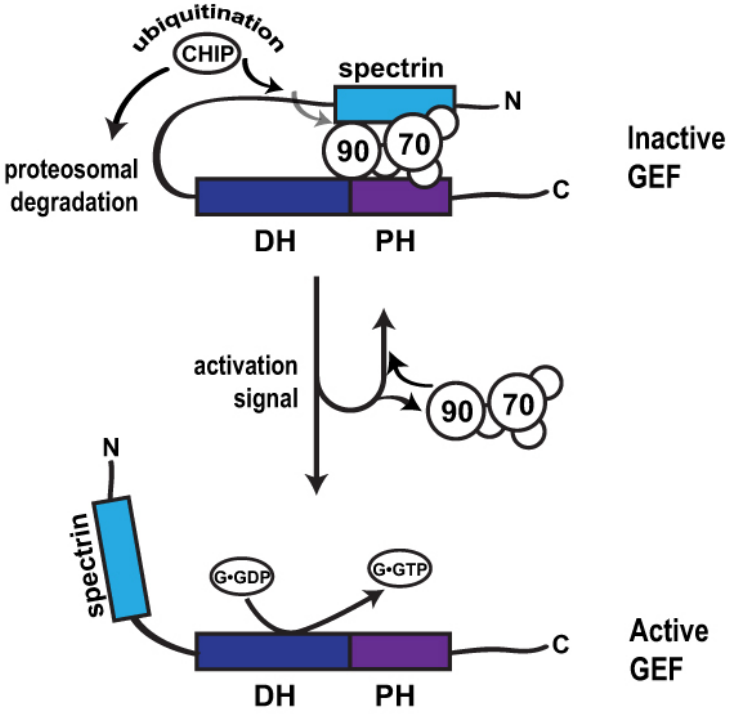

Figure 1-6. Regulation of proto-Dbl activity and stability by intracellular and intermolecular interactions. Proto-Dbl exists in an inactive or partially active state maintained by complex intra- and intermolecular interactions. The N-terminal spectrin domain indirectly associates with the PH domain through the Hsc70-Hsp90 chaperone complex. This interaction blocks the access to the catalytic DH domain, and masks the intracellular targeting function of the PH domain. Upon an activation signal, the closed inactive Dbl conformation is opened, Hsc70-Hsp90 chaperone complex is released and the GEF gets activated. Hsc70 and Hsp90 function together to stabilize Dbl by recognizing damages or misfoldings and convert the GEF to a functional conformation. When there are too many disorders in GEF's structure or conformation, which cannot be repaired or when Dbl is in excess, then Hsc70-Hsp90 cooperate with the CHIP co-chaperone, which directs the GEF to ubiquitin-mediated degradation by the proteasomal pathway. This prevents Dbl abberant signaling. Abbreviations: DH, Dbl homology domain; PH, Pleckstrin homology domain; 70, heat shock cognate protein Hsc70; 90, heat shock protein Hsp90; CHIP, chaperone-dependent E3 ubiquitin-protein ligase.

The Hsc70/Hsp90/CHIP machinery is a well known system, which maintains quality control in cells by recognizing misfolded or damaged proteins. It further assists in proteins conversion to a functional conformation (Hsc70-Hsp90 role) or directs them to ubiquitin-mediated

degradation by the proteasomal pathway (CHIP role) [181]. Since proto-Dbl displays a high oncogenic potential, this mode of regulation maintains its steady-state expression at a low level and keeps its localization and activity “in check” [180]. Thereby, proto-Dbl, like many GEFs, exists in an inactive or partially active state maintained by complex intra- and intermolecular interactions [178, 180]. This inhibitory conformation can be altered by upstream regulatory signals, which involve membrane targeting [182], oligomerization [183], tyrosine phosphorylation upon RTK stimulation [184], interactions with heterotrimeric G-protein subunits [185-187], protein kinases [184], adaptor or scaffolding proteins [188], as well as phosphoinositol phosphates [189], and result in intracellular translocation and stimulation of the GEF catalytic activity. *In vivo* proto-Dbl was shown to play a role in dendrite elongation. However, the normal development and function of brain and gonads, known to express the GEF, were not altered in Dbl-*null* mice, which were fertile and viable [190].

1.5.4. Dbl’s Role in Cancer

Although proto-Dbl overexpression is sufficient to cause cell transformation, the transforming potential of onco-Dbl is much higher and its expression has much more dramatic consequences for the cell [170]. Oncogenic Dbl activation is triggered by the truncation of N-terminal sequence of proto-Dbl including the spectrin module, which results in constitutive activation of the catalytic DH-PH core [168, 178]. Onco-Dbl escapes the control of the Hsc70/Hsp90/CHIP machinery, which translates into its increased stability and protection against ubiquitination and degradation [180]. Consistent with this, oncogenic Dbl becomes constitutively expressed, accumulates in the cells at high levels and in turn causes continuous activation of downstream GTPases and their signaling pathways leading to cell transformation [180]. Which events exactly trigger oncogenic Dbl formation is not known. Early studies, which led to onco-Dbl identification revealed that 5’ rearrangements of two separately isolated oncogenes (66 kDa and 76 kDa) were not detected in the original tumors, where the DNA for NIH3T3 transformation was isolated from, suggesting that oncogenic truncation might be triggered by gene transfer *in vitro* or is present only in a small minority of cells *in vivo* [166]. DNA fragments found in these tumors corresponded to fragments observed in normal human placenta DNA [166]. However, further efforts to study Dbl contribution in naturally occuring tumors were undertaken. Dbl expression was investigated in tumors of neuroectodermal origin. Proto-Dbl mRNA was detected in almost all Ewing’s sarcoma cell lines and tumors tested, as well as in lung metastasis of this cancer [191]. In contrast, proto-Dbl transcripts were not found in neuroepitheliomas and neuroblastomas suggesting its potential role as diagnostic marker of Ewing’s sarcomas [191]. Furthermore, proto-Dbl mRNA was not detectable in tumors of hematopoietic origin, but its preferential

expression in neuroectodermal and neuroendocrine tumors and also normal tissues of neuroectodermal origin was confirmed [192]. However, it was not found in gliomas, neuroblastomas (NB), Merkel's tumors and medulloblastomas, suggesting that proto-Dbl expression is not common for all neuroectodermal tissues, but only for some specific tissues of such origin [192]. Interestingly, it was shown that the expression level of proto-Dbl in the GEF-positive tumors is not high enough to cause cellular transformation in NIH3T3 fibroblasts. These results indicate the lack of a pathological role of proto-Dbl in malignant transformation [192]. In contrast to previous research, another group demonstrated wide expression of onco-Dbl not only in Ewing's sarcoma, but also in peripheral neuroectodermal tumor (PNET), NB, rhabdomyosarcoma, retinoblastoma and other tumors, arguing with the fact that Dbl can serve as good molecular marker in neuroectodermal cancer diagnosis [193]. Dbl oncogene functions were studied in the lenses of 2 days, 2 weeks, and 6 weeks old onco-Dbl transgenic mice [194]. DNA microarray analysis revealed interesting transcriptional profiles indicating the up-regulation of genes compatibile with epithelial-mesenchymal transition (EMT), angiogenesis and inhibition of apoptosis, which coincided with a strong disruption of lens structure and function [194]. Interestingly, the neuroepithelial tissue tumors in onco-Dbl transgenic mice developed only in the absence of functional p53 [195].

2. AIMS OF THE STUDY

The role of class II PI3KC2β in oncogenesis has just started to be appreciated. It was found to be involved in pro-survival and pro-migratory responses in human cancer cells, as well as in proliferation and protection against anoikis [103]. Its involvement in chemoresistance mechanisms was also described [139-141]. In terms of molecular mechanisms, there is not much known yet about PI3KC2β mode of activation, especially about the significance of its uncommon multidomain structure. However, a number of possible lipid- and protein-binding sites in the PI3KC2β sequence suggest sophisticated mechanism of regulation and a variety of downstream pathways, which may be triggered. The existing reports have described PI3KC2β assembly into large protein complexes and link the class II PI3K function to RhoGTPases and their associated activating proteins GEFs. RhoGTPases are crucial for cell migration and adhesion processes, and are frequently over-expressed in cancer leading to tumor cells invasion and metastasis. This makes RhoGTPases and GEFs promising targets for anti-cancer therapies.

Studies initiated in our lab had revealed that overexpression of PI3KC2β in NIH3T3 mouse fibroblast causes strong stress fibers assembly, increased membrane ruffling and cell spreading due to Rac1 and RhoA activation. The mechanism underlying these striking phenotypical changes revealed the involvement of Dbl GEF and shed a light onto the possible mechanism of PI3KC2β-mediated RhoGTPase activation, although the exact molecular mechanism remains elusive. Due to the high oncogenic potential of Dbl and increasing knowledge about PI3KC2β involvement in cancer, it was interesting to investigate what is the role of PI3KC2β in GEF-mediated RhoGTPases signaling.

Therefore, the aim of the first project was to translate the knowledge about PI3KC2β and Dbl structural features into a precise mechanism of action, as well as to further understand the functional role of the PI3KC2β/Dbl protein complex in the context of cytoskeletal remodelling in mouse fibroblasts.

Furthermore, the mechanism of PI3KC2β activation, regulation and resulting biological functions has never been studied in detail. Very little is known about PI3KC2β post-translational modifications, spatial and temporal organization upon cellular stimulation. Tyrosine phosphorylation of PI3KC2β was observed in different ligand-dependent contexts: for instance, in NIH3T3 and A-431 or HEK293 cells upon stimulation with PDGF and EGF respectively, as well as in SCLC and H-209 cells after treatment with SCF, HGF, insulin and FGF2 [77, 99]. However, detailed functional investigation of site-specific phosphorylations had never been carried out. We were therefore very much interested in defining the function of PI3KC2β tyrosine site-specific phosphorylation and its significance for the regulation of

PI3KC2β enzymatic activity, interactions with its binding partners and activation of downstream signaling pathways.

Thus, the aim of the second project was to identify and functionally characterize specific PI3KC2β tyrosine phosphorylation sites and signaling pathways, which they trigger.

3. RESULTS

3.1. Phosphoinositide 3-Kinase C2β Regulates RhoA and the Actin Cytoskeleton through an Interaction with Dbl (Project I)

3.1.1. Summary

The main function of PI3KC2β has been associated with cell migration and the related cytoskeletal remodeling processes. These cellular responses are mostly governed by Rho family of small GTPases. In these studies I have investigated the regulatory mechanism linking PI3KC2β to activation of Rho GTPases through the Dbl guanine exchange factor (GEF) in NIH3T3 mouse fibroblasts. PI3KC2β over-expression in NIH3T3 cells showed strong stress fibers formation, enlargement of the cell body, as well as increased cell spreading and ruffles formation. The results revealed formation of an endogenous PI3KC2β/Dbl complex in NIH3T3, as well as in neuroblastoma cancer cells. However, the observed association was neither dependent on the PI3K activity, nor on the EGF and PDGF stimulation, suggesting PI3KC2β/Dbl constitutive binding and a scaffolding role of the kinase in the complex with the GEF. Subsequently, PI3KC2β/Dbl interaction studies did not show a direct association between the N-terminal regulatory PI3KC2β domain and the spectrin- and pleckstrin homology (PH)-Dbl domains in the *in vitro* binding assay, suggesting a possible requirement for additional mediators of the interaction. Additionally, no difference in Dbl activity was observed in the *in vitro* GEF activity assay upon isolation of Dbl from HEK293 cells transfected with PI3KC2β wild-type and dominant-negative (kinase-dead) form. Therefore, a more complex mechanism of PI3KC2β-dependent Dbl activation is likely, which involves additional interaction partners. Moreover, an interaction of PI3KC2β isolated from A-431 epidermoid carcinoma cells with oncogenic form of Dbl has been found indicating a potentially important role of the kinase in Dbl-dependent tumorigenesis.

Results obtained for this project were accepted for publication in the *PLoS ONE* peer-reviewed journal:

Błajecka K, Marinov M, Leitner L, Uth K, Posern G, Arcaro A. Phosphoinositide 3-Kinase C2β Regulates RhoA and the Actin Cytoskeleton through an Interaction with Dbl. *PLoS ONE* (July 2012, accepted)

3.1.2. Introduction

The involvement of PI3KC2β in the cell migration and adhesion processes in the RTK- or GPCR-dependent manner was discovered in different cell line models, including cancer cells. Cell migration and adhesion mechanisms are controlled by Rho family GTPases and their associated modulating proteins such as GEFs, GAPs and GDIs. It was demonstrated that PI3KC2β can stimulate Rac activity in A-431 epidermoid carcinoma cells, which results in an increase in the cells migration speed [103]. This effect was mediated by the formation of a PI3KC2β multiprotein signaling complex downstream of EGFR involving Grb2 and Shc adaptor molecules, as well as the Eps8/Abi1/Sos1 complex, which plays a role of guanine nucleotide exchange factor for Rac. Furthermore, an association of PI3KC2β with the modular scaffold intersectin, which can also play a role of GEF, was described [104].

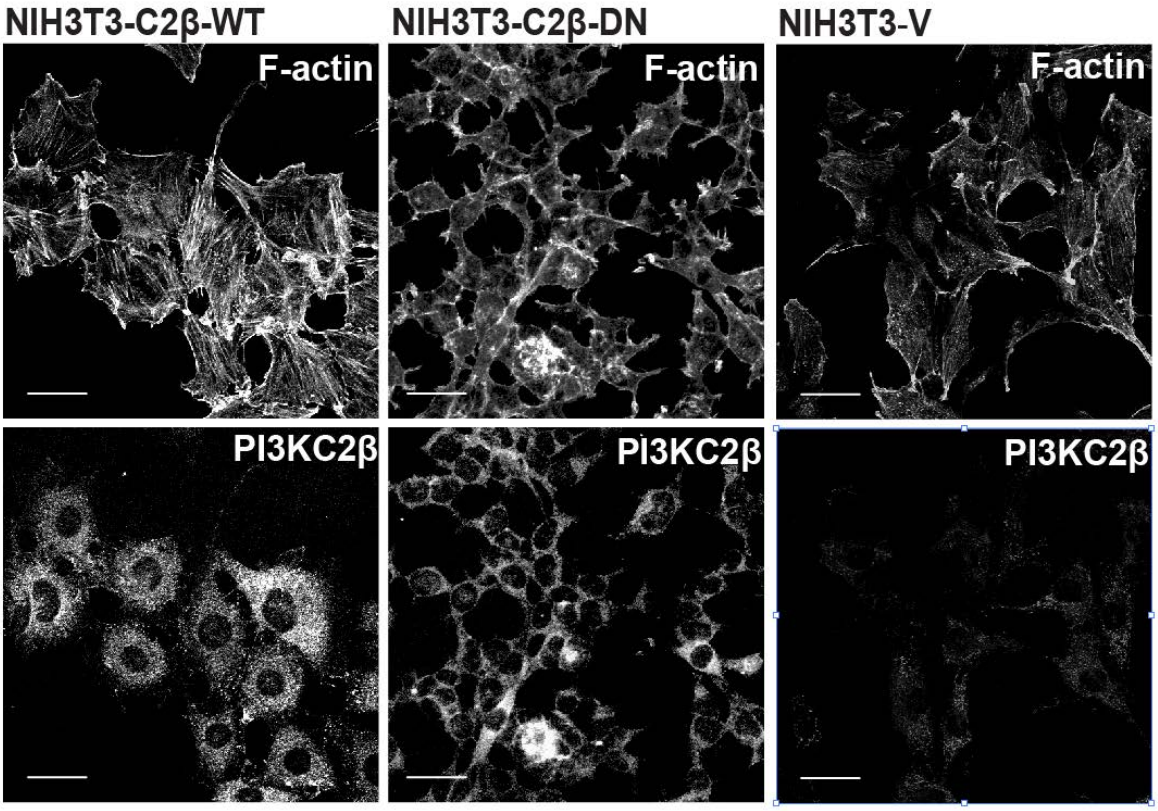

Figure 3-1. Kinase-dependent PI3KC2β effect on cytoskeletal rearrangements in NIH3T3 cells. Confocal images of NIH3T3 cells expressing PI3KC2β wild-type (-C2β-WT), kinase-dead dominant-negative (–C2β-DN) and the empty vector pcDNA3 (-V). Cells grown on cover slips for 24 h in 10% FCS and were stained with Alexa Fluor 555 dye to localise F-actin, and an anti-PI3KC2β antibody follwed by FITC-labelled anti-rabbit antibody, to localize the kinase. Scale bar represents 40 μm.

Preliminary results from our lab revealed that PI3KC2β plays a crucial role in controlling the actin cytoskeleton in NIH3T3 cells. Stable over-expression of PI3KC2β in these cells induced marked cell morphology changes in the cytoskeletal organization including increased cell spreading with strong stress fibers assembly (Blajecka *et* al. 2012, manuscript accepted) (Fig. 3-1). In contrast, the dominant-negative form of PI3KC2β strongly suppressed these phenotypical modifications. NIH3T3-C2β WT cells were much more enlarged in comparison to cells expressing the empty vector, while reduced cell size was observed in NIH3T3-C2β DN cell line (Fig. 3-2). Increased spreading of NIH3T3-C2β WT cells with strong stress fibers formation were associated with up-regulation of RhoA and Rac1 activity in 10% FCS and upon stimulation with growth factors (Blajecka *et* al. 2012, manuscript accepted). In order to investigate the molecular mechanism of PI3KC2β–dependent RhoGTPases activation in NIH3T3 cells, binding of PI3KC2β to a panel of RhoGEFs family members was examined. The Rho family GEF Dbl was identified as an interaction partner of PI3KC2β in NIH3T3 mouse fibroblasts over-expressing PI3KC2β. Grb2 adaptor protein was an additional player, which was found in the PI3KC2β protein complex. We were further interested in the precise mechanism of PI3KC2β activation leading to the observed phenotypical changes and what is the contribution of Dbl GEF in this process.

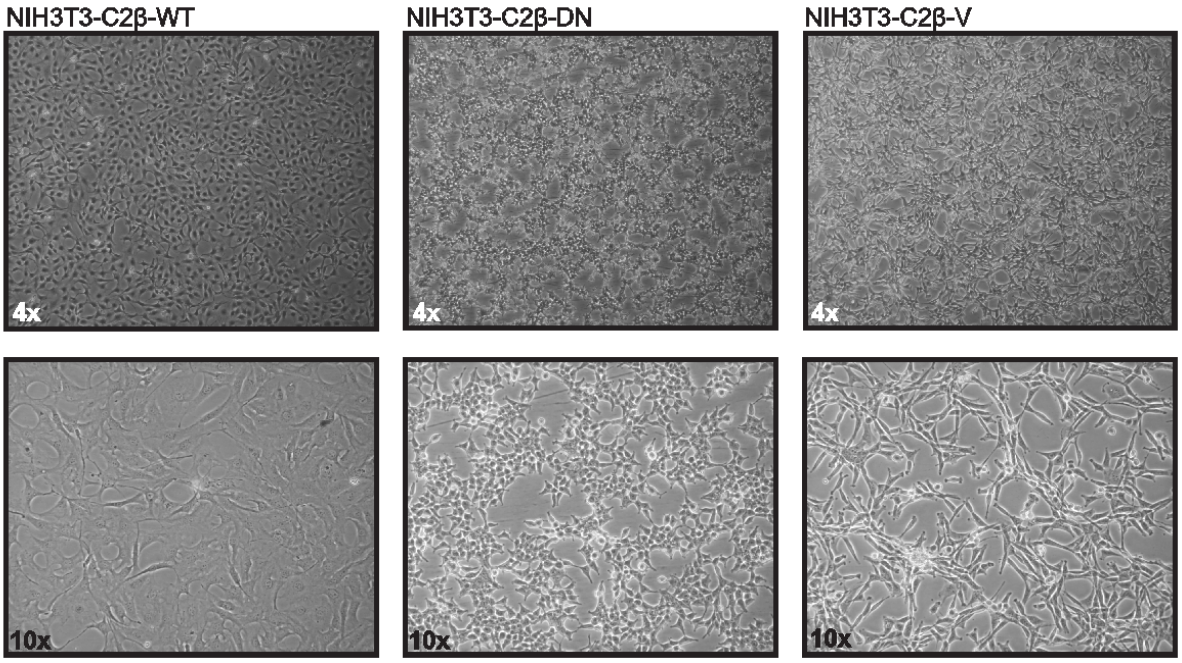

Figure 3-2. Cell size differences of PI3KC2β-WT and -DN stably transfected mouse fibroblasts. NIH3T3 cells expressing PI3KC2β wild-type (-C2β-WT), kinase-dead dominant-negative (–C2β-DN) and the empty vector pcDNA3 (-V) were seeded in DMEM complete medium supplemented with 0.8 mg/ml G418 selection antibiotic. 24 hrs after plating, morphology pictures were taken with Eclipse TS100 inverted microscope (4x and 10x magnification) supplied with a Nikon DXM1200 digital camera.

3.1.3. Results

Endogenous PI3KC2β interacts with Dbl in mouse fibroblast and human cancer cells

The Rho family guanine nucleotide exchange factor (RhoGEF) Dbl was identified as an interaction partner of PI3KC2β in the NIH3T3 mouse fibroblasts stably over-expressing the kinase. In order to investigate whether PI3KC2β and Dbl can form protein complex in physiological conditions I immunoprecipitated Dbl from the parental NIH3T3 cells and subjected it to western blot analysis with PI3KC2β-specific antibody. Results revealed PI3KC2β/Dbl complex assembly in mouse fibroblasts, although due to the low expression of the PI3KC2β in these cells the signal indicating the interaction was very weak (Fig. 3-3 A). To confirm this finding, we selected IMR5, a neuroblastoma cancer cell line, which expresses relatively high levels of PI3KC2β and Dbl. The importance of PI3KC2β for neuroblastoma tumorigenesis was recently published and the expression of oncogenic *dbl* in some tumors of neuroectodermal origin was investigated before [114, 193]. Thus, we performed co-immunoprecipitation with anti-Dbl and anti-PI3KC2β antibody in IMR5 cells and we found PI3KC2β association with Dbl, which confirmed the formation of an endogenous protein complex and the possible relevance of this interaction in the human cancer (Fig. 3-3 B).

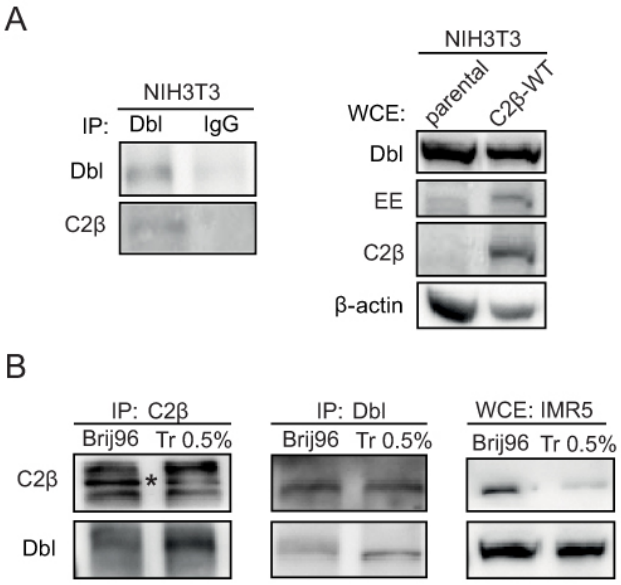

Figure 3-3. PI3KC2β interacts with Dbl GEF for Rho GTPases in mouse fibroblasts and human cancer cells. Complex formation of endogenous PI3KC2β and Dbl immunoprecipitated from the whole cell extract (WCE) of the parental NIH3T3 cells (A) and from the IMR5 neuroblastoma human cancer cells (B) with anti-Dbl, anti-C2β or control IgG antibody. In the case of IMR5 cell line Triton 0.5% and Brij96 1% lysis buffers were used, while NIH3T3 cells were lysed with Triton 1%. Samples were subjected to western blot analysis with the indicated antibodies. EE refers to anti-Glu-tag antibody. Asterisk indicates PI3KC2β.

In support of this result, lysis buffers of different strength were tested in order to examine the complex stability. PI3KC2β/Dbl binding was detected independently of the lysis buffer used (Brij96 or Triton 0.5%) demonstrating the stability of this interaction in the neuroblastoma cells.

Dbl interacts with the N-terminal domain of PI3KC2β and Grb2

To understand whether PI3KC2β directly influences Dbl activation, we were first interested in studying in detail whether the two proteins directly associate with each other and what is the molecular mechanism of PI3KC2β/Dbl complex assembly. In order to investigate whether PI3KC2β N-terminal and C2 domain C-terminal domains are involved in the interaction, we performed pull-down experiment using equimolar amounts of GST-fused PI3KC2β N-terminal and C2 C-terminal domains. Purified proteins were further incubated with lysates of HEK293 cells transfected with HA-tagged proto-Dbl, empty vector (V) or untransfected cells. Results revealed that the N-terminal domain of the PI3K is involved in the interaction with Dbl (Fig. 3-4 A). Interestingly, it associated to exogenous, as well as endogenous GEF indicating a physiological relevance of this interaction. The N terminus of PI3KC2β (in particular proline-rich regions) was previously shown to mediate PI3KC2β constitutive association with the EGFR through the SH3 domains of the Grb2 adaptor protein [94]. Moreover, PI3KC2β also interacted directly with one of five SH3 domains of intersectin, a multidomain scaffolding protein, which can also function as a guanine exchange factor [104]. On the other hand, Grb2 was shown to bind various GEFs [22]. It is therefore possible that Dbl binds to the PI3KC2β N-terminus through the Grb2 adaptor molecule. However, considering the domain structure of Grb2 and Dbl, it is unlikely that they bind directly. Further studies were therefore necessary to confirm that hypothesis. Pull-down experiments with purified GST-fused Grb2 and lysates of HEK293 cells transfected with HA-tagged proto-Dbl showed Grb2 binding to Dbl (Fig. 3-4 B). Grb2 complex formation with endogenous Dbl was detected in untransfected HEK293 cells, which again suggested the physiological relevance of the interaction. Previously published association of the adaptor molecule with PI3KC2β confirmed the accuracy of our results. All these findings demonstrate the formation of a multi-protein complex comprising PI3KC2β, Grb2, Dbl, which may be functional in untransformed cells (NIH3T3, HEK293), as well as in cancer cells (IMR5). Nevertheless, further experiments were required to investigate how Dbl structurally contributes to the interaction and whether the PI3KC2β and Dbl interactions in this complex are direct or mediated by Grb2 or other possible interaction partners.

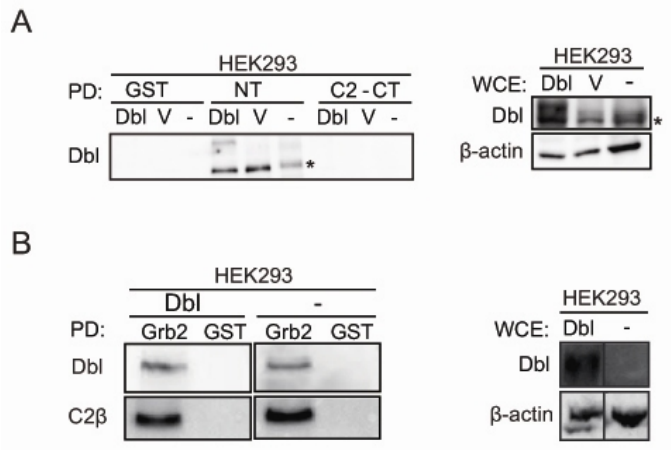

Figure 3-4. Dbl interacts with N-terminal PI3KC2β domain and Grb2. (A) Interaction between the PI3KC2β and Dbl was examined by the pull-down (PD) of PI3KC2β GST-fused N-terminal and C2 C-terminal domains in HEK293 cells transfected with HA-proto-Dbl (Dbl), pcDNA3 empty vector (V) or untransfected control (-). Complex formation was shown by immunoblotting with anti-Dbl antibody. (B) Interaction between the Dbl and Grb2 was examined by the GST-Grb2 pull-down in HA-proto-Dbl (Dbl) transfected and untransfected (-) HEK293 cells. Pull-down samples were subjected to western blot analysis with indicated antibodies. Asterisk indicates endogenous Dbl.

Dbl spectrin- and PH- domains bind to PI3KC2β in COS-1 cells

Considering the domain structure of Dbl and the complex mechanism of GEFs activation and regulation, we were further interested in which part of the Dbl protein sequence is involved in the interaction with PI3KC2β. For that reason we co-transfected COS-1 cells with GST-fused Dbl N-terminal-, spectrin-, onco-, PH- domains and PI3KC2β wild-type and subjected the lysates to pull-down experiments with glutathione-sepharose beads. A schematic representation of Dbl mutants is shown on Fig. 3-5 A. Western blot analysis of pull-down samples revealed a prominent interaction of the PI3KC2β with the Dbl spectrin- and PH- domain (Fig. 3-5 B). These results go in line with previous reports describing heat shock cognate protein 70 (Hsc70) as a binding partner of the Dbl amino-terminal spectrin homology- and PH- Dbl domains. These interactions result in Dbl adopting an inactive conformation, which on one hand limits the GEF's catalytic activity by blocking an access of RhoGTPases to its DH- catalytic domain, and on the other hand restrain Dbl intracellular distribution [179]. However, it is just one aspect of the complicated Dbl regulation machinery and it is difficult to state whether PI3KC2β plays a similar role to Hsc70 in regulation of Dbl activity. Interestingly, a weak interaction of the N-terminal domain of Dbl with the kinase was observed even though it includes the spectrin domain, which on its own was quite strongly bound to PI3KC2β (Fig. 3-5 B). Similarly, the PI3K association to onco-Dbl, which contains the PH domain, was either not detectable or very faint in different repetitions of the experiment (Fig. 3-5 B). Interestingly, no interaction of endogenous Grb2

was observed with any of the Dbl domains in COS-1 cells, suggesting that neither does Grb2 bind to Dbl directly, nor does it mediate PI3KC2β association with the Dbl domains. However, when Dbl was purified as a full-length protein, a weak interaction with Grb2 could be identified. It is therefore likely that the full-length of GEF is needed for an interaction with Grb2 and other possible mediators of the association with PI3KC2β. Further investigations were conducted to assess the possible contribution of PI3KC2β to the regulation of Dbl activity and function. It is possible that PI3KC2β may compete with Hcs70 and other associated proteins for binding to Dbl. This may induce Hsc70-Hsp90 complex disassembly and opening of the GEF inactive conformation resulting in Dbl activation. Whether PI3KC2β and Dbl protein-protein interactions are direct or are mediated by other possible binding partners was subsequently elucidated in an *in vitro* binding assay.

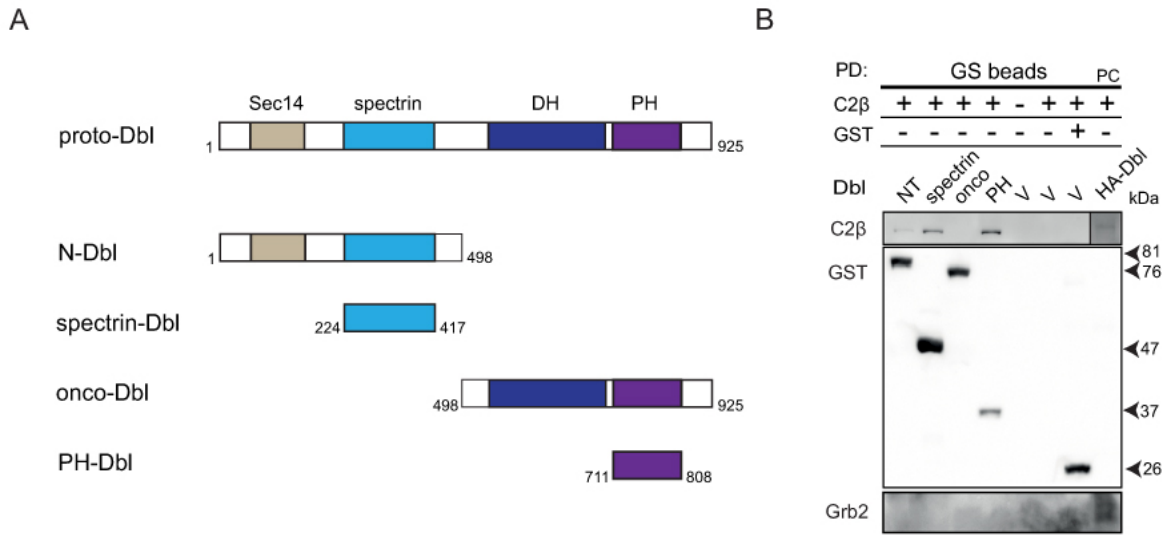

Figure 3-5. PI3KC2β binds with high affinity to the spectrin- and PH- Dbl domain. (A) Schematic representation of Dbl mutant constructs, which were a kind gift of Prof. Danny Manor from the School of Medicine at Case Western Reserve University, Cleveland, USA. (B) COS-1 cells co-transfected with Myc-PI3KC2β and different GST-fused Dbl domains (N-, spectrin-, onco-, PH-) in pEBG vector were subjected to gluthatione-sepharose beads affinity-purification and immunobotted with indicated antibodies. PC refers to positive control that are COS-1 cells co-transfected with PI3KC2β and HA-proto-Dbl, and immunoprecipitated with anti-Dbl antibody.

Dbl spectrin- and PH- domains do not associate directly with N terminus of PI3KC2β and Grb2

The binding of Dbl spectrin and PH domains to PI3KC2β in co-transfected COS-1 cells led us to test whether the proteins interact directly with each other or are associated in the complex via other interaction partners. Since it was known from previous pull-down assays that Dbl binds to the N-terminal domain of PI3KC2β and Grb2, we performed an *in vitro* binding assay, which utilized soluble N-terminus of PI3KC2β or/and Grb2, which were previously

expressed and purified from *E.coli* as GST-fused proteins. Next, purified proteins were treated with thrombin to remove GST-tag in order to avoid unspecific dimerization with GST-tagged Dbl domains. Soluble NT-PI3KC2β and Grb2 were further incubated with recombinant GST-fused spectrin- and PH- Dbl domains expressed and purified from COS-1 cells, and immobilized on gluthatione-sepharose beads (Fig. 3-6 A). In the case of NT-PI3KC2β, we did not observe a direct interaction with Dbl mutants. On the other hand, Grb2 showed a weak interaction with Dbl spectrin–domain and stronger association with PH-Dbl fragment. The binding was observed in both reactions, when Grb2- was incubated with the Dbl domains alone and when co-incubated with NT-PI3KC2β. However, due to a detectable interaction of GST control with soluble NT-PI3KC2β and Grb2- we were cautious in iterpreting this data as specific. Increasing the reaction buffer strength in the *in vitro* experiments (from Brij96 to Triton 1%) did not help to reduce the unspecific signal. To demonstrate the accuracy of the performed *in vitro* reactions with Dbl mutants, control reactions with known-to-bind ptoteins (NT-C2β-GST + Grb2-; Grb2-GST + NT-C2β-) were performed exactly in the same way. The control experiment showed the expected interactions without any undefined signals indicating accuracy of our experimental procedure (Fig. 3-6 B).

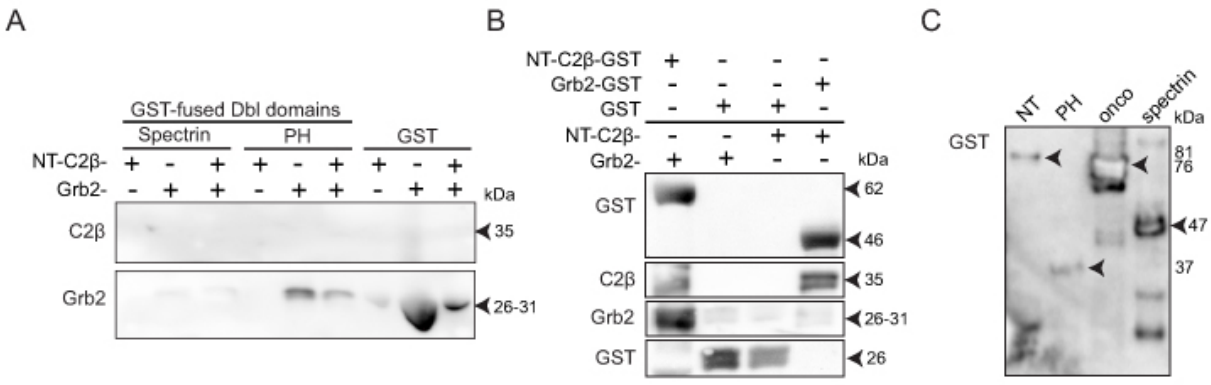

Figure 3-6. Lack of Dbl direct association with NT-PI3KC2β and Grb2. (A) GST-tagged and gluthatione-sepharose beads conjugated spectrin- and PH- Dbl domains, and GST as control, were incubated *in vitro* with GST-truncated soluble PI3KC2β N-terminal domain (NT-C2β-) (~35 kDa) and Grb2 (26-31 kDa). To detect PI3KC2β and Grb2 interaction wih Dbl domains samples were subjected to immunoblotting with indicated antibodies. The same results were obtained in three experiments independently of the buffer used (Brij96 or Triton 1%). One representative blot is shown (Brij96 1% was used for the incubation). (B) Control *in vitro* reactions, which utilized immobilized GST-tagged NT-C2β domain, Grb2 and GST alone, and soluble GST-truncated NT-C2β- domain and Grb2-, were performed. Samples were subjected to SDS-PAGE analysis and the interaction between NT-C2β and Grb2 was detected with indicated antibodies by western blot analysis. (C) Representative immunoblot of Dbl domains separated on SDSPAGE and analysed by wester blot with anti-GST antibody.

The unspecific binding in the negative GST control of the *in vitro* reaction, the lack of potential interacting motifs in Grb2 and Dbl structure, and the fact that we did not observe an interaction of endogenous Grb2 with any of the Dbl domains in the gluthatione-sepharose beads pull-down in COS-1 cells co-transfected with Dbl mutants and PI3KC2β (Fig. 3-5 B), let us to believe that the *in vitro* interaction of Grb2 with spectrin- and PH- Dbl domain is not reliable. Moreover, the lack of a direct association of NT-PI3KC2β with spectrin- and PH- Dbl domains *in vitro* suggests that other interaction partners, but not Grb2 adaptor protein, are required for the association of the PI3K with the GEF in living cells.

PI3KC2β isolated form A-431 human cancer cells binds to onco-Dbl domain

The indirect PI3KC2β binding to Dbl was demonstrated in NIH3T3, HEK293 and additionally in the human cancer cell line IMR5. The detailed interaction with spectrin- and PH- Dbl domains was further investigated in COS-1. No association was found with the oncogenic Dbl. However, all my experiments aiming at investigation of PI3KC2β/Dbl domain-specific interactions were performed so far in non-cancerous cell systems such as COS-1 or HEK293 cells, which may certainly not reflect the situation in cancer cells at the molecular and functional level. Therefore, I purified PI3KC2β from A-431 epidermoid carcinoma cell line overexpressing PI3KC2β WT (A-431-C2β) and subjected it for the pull-down with lysates of COS-1 cells transfected with Dbl domains, which turned out to be the best system for Dbl mutants' expression. In A-431-C2β cells the kinase associated with Eps8/Abi1/Sos1 complex and was localized in the cytosol, but also in the actin cytoskeleton, particularly in cell protrusions and ruffling regions of the plasma membrane at the edges of cells colonies [103]. Surprisingly, my results showed that PI3KC2β purified form A-431-C2β cells bound only to onco-Dbl mutant expressed in COS-1 cells, but not to the spectrin- and PH- domain (Fig. 3-7 A), as I observed in COS-1 cells co-transfected with PI3KC2β and Dbl mutants (Fig. 3-5 B). Unexpectedly, it did not bind to NT- and spectrin- domain, or to PH-Dbl (Fig. 3-7 A), suggesting that proteins possibly immunoprecipitated with PI3KC2β from A-431-C2β cells function in the complex, which preferentially interact with onco-Dbl than with proto-Dbl characteristic domains. It is therefore possible that in cancer cells different molecular mechanism exists, which facilitate PI3KC2β binding with oncogenic form of Dbl rather than proto-Dbl. It other words, in some cancers carrying an N-terminal domain Dbl truncation mutation, PI3KC2β potentially may play an important role in the Dbl-dependent tumorigenesis.

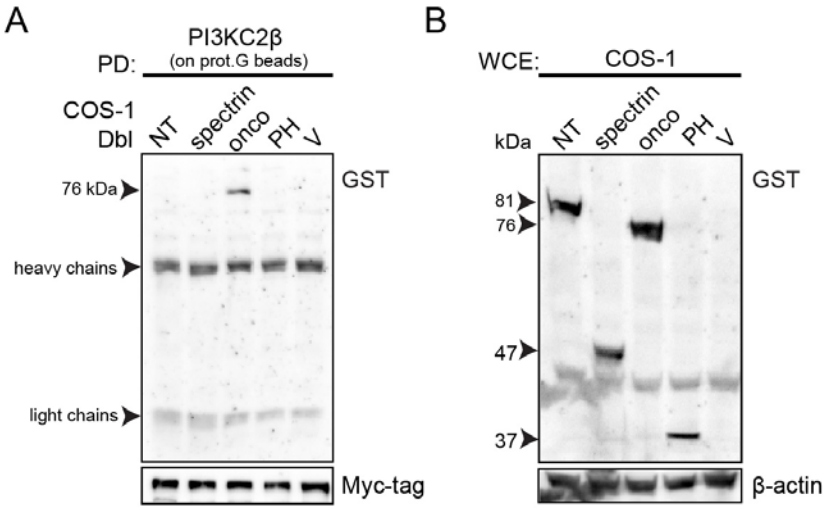

Figure 3-7. PI3KC2β interact with onco-Dbl when purified from cancer cells. Western blot analysis representing pull-down experiment (PD) of Myc-tagged PI3KC2β, immunopurified from A-431 cells over-expressing PI3KC2β and immobilized on Protein G beads (A), with whole cell extracts (WCE) of COS-1 cells transfected with Dbl mutants, namely NT-, spectrin-, onco- and PH- doamins (B). Transfection efficiency and interaction of PI3KC2β with onco-Dbl was studied with indicated antibodies.

The assembly of the PI3KC2β/Dbl complex is not modulated by PI3K activity or cell stimulation with EGF or PDGF

The data presented above demonstrated PI3KC2β complex formation with Dbl, which potentially explains modulation of Rho/Rac activity in mouse fibroblats over-expressing the kinase, and results in the kinase-dependent enlargement of the cell body, generation of the stress fibers and ruffles. Although an *in vitro* binding assay did not show a direct or Grb2-mediated PI3KC2β association with GEF, it did not exclude a role for the PI3KC2β catalytic activity in the control of Dbl. In order to further investigate the significance of PI3KC2β kinase activity for its interaction with Dbl, we assessed the ability of PI3KC2β WT and DN to interact with Dbl in transiently transfected HEK293 cells and stably transfected NIH3T3 cells. In HEK293 cells, both PI3KC2β WT and DN interacted with endogenous or ectopically expressed (HA-tag) Dbl in a similar manner (Fig. 3-8 A). This observation was confirmed in NIH3T3-C2β-WT and NIH3T3-C2β-DN cells (Fig. 3-8 B), that suggested lack of the PI3KC2β kinase activity requirement for complex formation with Dbl.

Results form our lab showed increased Rho/Rac activity in NIH3T3 cells over-expressing the kinase upon growth factor stimulation (Blajecka *et* al. 2012, manuscript accepted). Therefore, we next assessed whether the PI3KC2β/Dbl complex was constitutively assembled, or induced by cell stimulation with polypeptide growth factors. The PI3KC2β/Dbl interaction could be detected in serum-starved NIH3T3-C2β-WT cells, and the

interaction was apparently not increased by cell stimulation with EGF or PDGF from 5' up to 30' (Fig. 3-8 C). Phosphorylation of extracellular signal-regulated kinase (Erk) was examined on cell lysates as growth factors stimulation control.

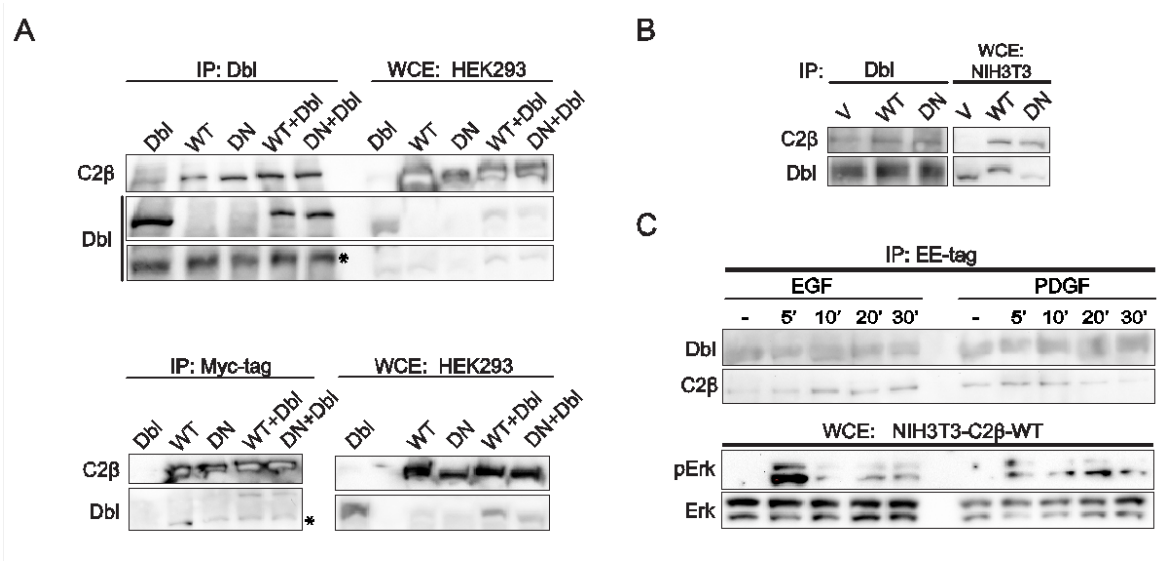

Figure 3-8. PI3KC2β kinase activity and growth factor stimulation is not required for complex formation with Dbl. (A) HEK293 cells were transfected with vectors encoding Dbl in combination with Myc-PI3KC2β WT or DN, or empty vector. Immunoprecipitates prepared with anti-Dbl or anti-Myc-tag antibodies were analysed by western blot with the antibodies indicated. (B) Lysates from NIH3T3-V, -C2β-WT or -C2β-DN cells were immunoprecipitated with anti-Dbl antibodies and analysed by western blot for an interaction with Dbl. (C) NIH3T3-C2β-WT cells were serum-starved (0.5% FCS) for 16 hrs, and were stimulated with EGF (20 ng/ml) or PDGF (20 ng/ml) for the indicated lengths of time. Immunoprecipitates prepared with anti-Glu (EE) tag antibodies were analysed by western blot with the antibodies indicated. Phosphorylation of Erk was investigated as growth factor stimulation control.

We finally investigated whether the catalytic activity of PI3KC2β contributes to the activation of Dbl GEF activity. In transiently transfected HEK293 cells, ectopic expression of PI3KC2β WT or DN did not alter the GEF activity of endogenous Dbl towards RhoA (Fig. 3-9). Taken together, these results indicate that PI3KC2β lipid kinase activity is not required for its association with Dbl, and does not modulate Dbl activity measured *in vitro*.

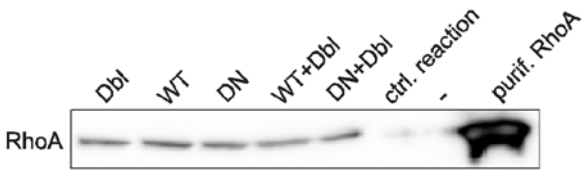

Figure 3-9. PI3KC2β does not modulate Dbl activity *in vitro*. HEK293 cells were transfected with vectors encoding Dbl in combination with Myc-PI3KC2β WT or DN, or empty vector. Immunoprecipitates prepared with anti-Dbl antibody were analysed for GEF activity towards recombinant RhoA.

3.1.4. Material and Methods

Reagents and Antibodies

The following antibodies were used: PI3KC2β was described in [72]**,** 9E10 myc epitope, Dbl, Grb2, RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Src (Epitomics, CA, USA); β-actin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland); donkey anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (1:10000 dilution) coupled with horseradish peroxidase (Amersham Biosciences). Anti-EE antibody was kindly provided by Julian Downward (CRUK). Alexa Fluor 555 was obtained from Invitrogen. Recombinant EGF, PDGF BB were purchased from Calbiochem, La Jolla, CA, USA. Guanosine 5'-diphosphate sodium salt (GDP) and guanosine 5'-triphosphate sodium salt (GTPγS), thrombin and p-aminobenzamidine-agarose were purchased from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.

Cell Culture

Mouse NIH3T3 fibroblast, HEK293 (human embryonic kidney 293), COS-1 (African green monkey kidney fibroblast-like) and A-431 epidermoid carcinoma cells over-expressing PI3KC2β wild-type were grown in DMEM medium (Life Technologies/Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% of L-glutamine (v/v), and were passaged every 3 to 5 days by trypsinization. Cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Stably transfected NIH3T3 clones and A-431 over-expressing PI3KC2β were grown in the presence of 0.8 mg/ml G418 or 1 mg/ml G418, respectively. NIH3T3 cell lines stably expressing Glu-tagged PI3KC2β wild-type (WT) and A-431 cells stably expressing Myc-tagged PI3KC2β wild-type (WT) were described previously [77].

Plasmids

The cDNA constructs encoding NH_2-terminal Glu- (MEFMPME) or Myc- (MEQKLISEEDL) epitope tags and dominant-negative kinase-dead PI3K-C2β (DN) were described previously [72, 99, 103]. HA-tagged proto-Dbl wild-type and GST-fused Dbl mutant constructs were a kind gift of Prof. Danny Manor from the School of Medicine at Case Western Reserve University, Cleveland, USA, and were described in [179]. Constructs encoding GST-PI3KC2β N-terminal and C2 C-terminal domains, GST-Grb2 and RhoA-CA-GST(V14) were purchased from Addgene repository. Construct of GST-rhotekin in pGEX-2T vector was a kind gift of Prof. Pontus Aspenström from the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet, Stockholm, Sweden.

Transient Transfections

HEK293 cells were transiently transfected with a Ca^{2+} phosphate protocol [72], while NIH3T3 and COS-1 cells were transfected with Lipofectamine 2000 or Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions.

Growth Factor Stimulations

NIH3T3 cells stably expressing PI3KC2β wild-type (WT) were grown to confluency and starved overnight (16 hrs) in DMEM containing 0.5% FCS and penicillin/streptomycin/L-glutamine. Cells were then stimulated with the EGF (20 ng/ml) or PDGF (20 ng/ml) for the indicated lengths of time. Cellular lysates were prepared as described below.

Cell Lysis

NIH3T3, COS1, HEK293 and A-431-C2βWT cellular lysates were prepared in Triton 1% buffer (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5% NP-40) supplemented with 7 x concentrated stock of Complete Mini Protease Inhibitor Coctail (Roche Applied Sciences) and with the phosphatase inhibitors: sodium fluoride (1 mM NaF), sodium ortho-vanadate (1 mM Na_3VO_4) and β-glycerophosphate (10 mM). For the studies of endogenous complex formation in IMR5 cells Triton 0.5% and Brij96 1% lysis buffers (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Brij96) supplemented with 7 x concentrated stock of protease and phosphatase inhibitors were used, as indicated in the figure legends. Cell pellet was removed by centrifugation for 30 min. (15,000 rpm at 4°C). The lysates were equalized for protein content with the Thermo Scientific Pierce BCA Protein Assay Kit and subjected to immunoprecipitation or loaded on SDS-polyacrylamide gel (SDS/PAGE) as a whole cell extracts (WCE). Prior loading on the gel, samples were supplemented with SDS sample buffer (50 mM Tris.HCl, pH 6.8, 2% or 5% SDS, 10% glycerol, 200mM DTT, and 0.25% bromophenol blue) and denatured for 5 min. at 95°C.

Western Blot

Proteins were separated by SDS/PAGE electrophoresis and immunoblotted on polyvinylidene fluoride membrane PVDF (Amersham, GE Healthcare, UK). Membranes were then blocked in 5% non-fat dry milk in 1x phosphate-buffered saline (PBS) over-night at 4°C. Washings were done in 1x PBS-Tween (0.1%). Incubation with the primary antibodies (diluted according to the manufacturer's protocol) was performed in 4°C over-night, and with the secondary antibodies 1 h at room temperature. Chemiluminescence was detected using

SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Immunoprecipitation

Cells were grown to confluence in 10-cm dishes, washed with ice cold 1x PBS before lysis on ice for 20 min. in 0.5-1 ml of Triton 1% lysis buffer (as described above). Insoluble material was removed by centrifugation for 30 min. (15000 rpm at 4°C). Immunoprecipitation with primary antibodies (diluted according to the manufacturer's instructions) was conducted on cell lysates equalized for protein content. Incubation with the respective primary antibodies was performed at 4°C for 2 hrs. Protein A or G Sepharose 4 Fast Flow beads (Amersham, GE Healthcare, UK) were then added respectively and the incubation was continued for 1 h at 4°C. Immunoprecipitates were washed three times in the lysis buffer and resuspended in 2x SDS sample buffer (50 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 200mM DTT, and 0.25% bromophenol blue). Samples were denatured for 3-5 min. at 95°C and analysed by SDS/PAGE and western blot.

Production of GST-Fusion Proteins

GST-fused N-terminal and C2 C-terminal domains of PI3KC2β, Grb2-GST, rhotekin-GST, RhoA-CA-GST(V14) in pGEX-2T expression vectors were propagated in *Escherichia coli* BL21 cells. Incubation proceeded at 37°C until OD_{600} reached 0.5. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and continued at 30°C for 3-4h. Bacteria were collected by centrifugation and resuspended in lysis buffer. For purification of GST-PI3KC2β-NT, –CT-C2 and GST-Grb2 following lysis buffer was used: 50 mM Tris HCl pH 7.5, 150 mM NaCl, 5mM EDTA pH 8.0 and 1% Triton supplemented with 7 x concentrated stock of Complete Mini Protease Inhibitor Coctail (Roche Applied Sciences) and with the phosphatase inhibitors (1 mM PMSF, 10 µM leupeptin, 10 µM pepstatin, 5 mM benzamidin, 1 mM DTT, 1 µl/ml aprotinin, 1 mM NaF, 1 mM Na_3VO_4). For purification of rhotekin-GST and RhoA-CA-GST(V14) lysis buffer was changed to: 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF. Bacteria were thereafter sonicated on ice 3 times for 45 sec with 1 min. intervals. The lysed cells were clarified by centrifugation at 15000 for 30 min. at 4°C. GST-fusion proteins were affinity-purified from the resulting supernatants by addition of glutathione-sepharose beads (Amersham Biosciences, Upsalla, Sweden) followed by incubation at 4°C for 60 min. The glutathione-sepharose beads were collected by centrifugation (2000 rpm) at 4°C followed by four washes with washing buffer: for GST-PI3KC2β-NT and –CT-C2 and Grb2 lysis buffer was used, and for the rhotekin-GST and RhoA-CA-GST(V14) buffer containing 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT was employed. After the final wash,

the beads containing GST-PI3KC2β-NT and –CT-C2 and Grb2 proteins were resuspended in washing buffer supplemented with 50% glycerol and were stored in -20℃. Rhotekin-GST was resuspended in washing buffer supplemented with 10% glycerol. The beads were divided into aliquots of 250 µl and stored in -80℃. RhoA-CA-GST(V14) was cleaved from the GST by thrombin digestion, treated with p-aminobenzamidine-agarose to remove thrombin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), snap-frozen and stored in liquid nitrogen. The purity of the GST-fusion proteins was assayed by SDS-PAGE.

GST-tagged NT-, spectrin-, onco- and PH- Dbl mutants were expressed in COS-1 cells, lysed with HEPES buffer like previously decribed [179] and purified with glutathione-sepharose beads (GE Healthcare). Beads containing GST-fusion proteins were washed 3-4x with ice-cold HEPES buffer and 2x with washing buffer (50 mM Tris pH=7.5, 150 mM NaCl). Next, they were resuspended in the washing buffer supplemented with 1mM DTT and 50% glycerol and stored in -20°C.

Pull-down Assays for PI3KC2β/Dbl/Grb2 Interaction

After 48 h of culture, untransfected and transfected with HA-proto-Dbl or pcDNA3 empty vector HEK293 cells were washed once with ice-cold 1xPBS. Cell extracts were prepared as described above. Protein concentration was determined (Thermo Scientific Pierce BCA Protein Assay Kit) and equal volume of lysates were immediately supplemented with equimolar amounts of GST-NT-PI3KC2β, GST-(CT)C2-PI3KC2β or GST-Grb2 immobilized on gluthatione beads. In the case of pull-down in COS-1 cell line, cells were co-transfected with wild-type PI3KC2β and GST-tagged Dbl domains (NT-, spectrin-, onco-, PH) or pcDNA3 empty vector and HA-proto-Dbl as control. 48 h post-transfection cell extracts were prepared as described above and equal amounts of lysates were supplemented with gluthatione-sepharose beads (Amersham Biosciences, Upsalla, Sweden) or PI3KC2β wild-type immobilized on Protein A-Sepharose beads and immunoprecipitated from A-431 cells overexpresing the kinase. Pull-down reactions were then incubated with constant rotation for 2 h at 4°C. Beads were washed four times with ice-cold lysis buffer. 2 x SDS-PAGE sample buffer was added to the beads. Samples were heated at 95°C for 3 min. and subjected to SDS-PAGE and western blot analysis.

In Vitro Binding Assay

GST-fused Dbl spectrin- and PH-domains proteins and GST alone were incubated *in vitro* with soluble Grb2- and NT-PI3KC2β- domain in 1% Brij 96 lysis buffer (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Brij96) supplemented with 7 x concentrated stock of Complete Mini Protease Inhibitor Coctail (Roche Applied Sciences) and phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate). Soluble

Grb2- and NT-C2β-domain were obtained from GST-fused Grb2 and NT-PI3KC2β domain as a result of digestion with thrombin followed by p-aminobenzamidine-agarose treatment to remove the enzyme (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). After constant rotation for 2 h, at 4°C, the complexes were washed 3 x with Brij 96 1% lysis buffer. 2 x SDS-PAGE sample buffer was added, samples were heated at 95°C for 3-5 min. and subjected to SDS-PAGE followed by western blot analysis.

GEF Activity Assay

HEK293 cells were transiently transfected with plasmids as described above. Cell lysates were prepared as above and immunoprecipitated with anti-Dbl antibody and Protein A-Sepharose beads. The immunoprecipitates were washed twice with lysis buffer and once with GEF reaction buffer (20 mM Tris-HCl pH 7.6, 0.5% Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and resuspended in GEF reaction buffer on ice. The reaction was started by the addition of 1 mM GTPγS and 50 µg/ml purified recombinant GDP-loaded RhoA, which was obtained as described previously [196]. Following 30 min. incubation at room temperature, samples were placed on ice and supernatants collected. Active RhoA was isolated from the supernatants by using GST-rhotekin, which was incubated together with lysates by constant rotation at 4°C for 30 min. The beads were thereafter washed 4x with ice-cold washing buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, µM leupeptin, 10 µM pepstatin and 1 mM PMSF) and analysed by SDS-PAGE and western blot.

Microscopy

NIH3T3 cells stably transfected with PI3KC2β wild-type (WT), kinase-dead dominant-negative (DN) and the empty pcDNA3 vector (V) were seeded in DMEM complete medium supplemented with 0.8 mg/ml G418 selection antibiotic. 24 hrs after plating morphology pictures were taken with Eclipse TS100 inverted microscope (4x and 10x magnification) supplied with a Nikon DXM1200 digital camera.

3.1.5. Discussion

PI3KC2β role in the complex formation with Dbl

The analysis of PI3KC2β interacting molecules in NIH3T3 fibroblasts revealed a novel signalling complex implicated in RhoA and Rac1 activation. We identified the RhoGEF Dbl as a novel interaction partner of PI3KC2β and we hypothesise that, in collaboration with Dbl, PI3KC2β can modulate RhoA and Rac1 activity and thus control cytoskeletal rearrangements. Dbl is a crucial RhoGEF with activity towards Cdc42, RhoA and Rac1 [172]. It was also the first mammalian RhoGEF to be identified [165], but the mechanism of its activation is still not completely understood. In order to relieve its intrinsic auto-inhibitory activity, a physical interaction between the GEF's N-terminal spectrin- and C-terminal PH-domain has to be released. This change in the inactive conformation of the protein can be triggered by diverse regulatory mechanisms including phosphorylation, or interactions with other proteins. In my studies, the prominent binding of PI3KC2β to the spectrin- and PH-domain of Dbl was similar to the results reported for Dbl binding to the chaperone Hsc70 [179] (Fig. 3-5 B). Hsc70 together with Hsp90 and the ubiquitin ligase CHIP keep Dbl activity in check by either stabilising its inactive conformation or directing the GEF to proteosomal degradation [180]. How PI3KC2β contributes to Hsc-70-Hsp90-CHIP machinery is an interesting issue. My results concerning Dbl domains interactions with the kinase supported the hypothesis that PI3KC2β may compete with Hcs70 and other Dbl-associated proteins for binding to the GEF. In turn, it could induce Hsc70-Hsp90 complex disassembly and opening of the GEF inactive conformation resulting in Dbl activation. This model of the Dbl activation mechanism was also suggested by Bi *et* al. [178], who showed that protein factors, associated to the actin stress fibers, can bind to Dbl PH domain and compete with the N-terminal sequences, leading to release of N-terminal constraint. However, an *in vitro* examination of the molecular mechanism of PI3KC2β/Dbl complex formation revealed that neither the N-terminal sequence of the PI3K, nor Grb2, directly bound to Dbl, although both molecules were present in the PI3KC2β complex isolated from living cells (Fig. 3-6). Formation of the Dbl/PI3KC2β complex was observed in serum-starved cells and the association was not further promoted by EGF or PDGF stimulation (Fig. 3-8 C). In addition, no evidence was found for a role of PI3KC2β in the activation of Dbl GEF activity, at least when measuring its activity *in vitro* (Fig. 3-9). It is therefore convincing that PI3KC2β plays a different role than direct regulation of Dbl activity in the protein complex. Other molecular mechanims involving the kinase and GEF must be involved for an increased Rho/Rac activation and observed phenotypes in the NIH3T3 cells.

PI3KC2β role in Dbl activation

The biological activity of proto-Dbl is in fact dependent on a combination of mechanisms that involve not only intramolecular interactions, but also N- and C-terminal domain-dependent turnover of the protein, and the PH domain binding to PtdIns [182]. It is possible that PI3KC2β alters the intracellular localization of Dbl by generating PtdIns(3)P or PtdIns(3,4)P_2 [72], which are considered as the main lipid products of the kinase. PI3KC2β potential to generate PI(3,4,5)P3 was shown only in the presence of equimolar amounts of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Moreover, the activity of the PI3KC2β toward PtdIns(4,5)P_2 was only about 1% of the whole PI3K activity under this conditions [72]. Interestingly, the Dbl PH domain was shown to not bind to PI3KC2β substrates (PtdIns and PtdIns(4)P) and product (PtdIns(3)P) *in vitro*, whereas the binding to PtdIns(3,4)P_2 product was not studied [189]. In contrast, the PH domain of Dbl associated with PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, and caused marked inhibiton of Dbl GEF activity, which was directly dependent on phospholipids concentration [189]. PtdIns and PtdIns(3)P did not have a significant effect on Dbl-mediated GDP dissociation from Cdc42 [189]. It would be therefore interesting to check whether the PI3KC2β product PtdIns(3,4)P_2 can be of any significance in triggering of proto-Dbl open, active conformation. However, it is important to mention here that according to the current knowledge, PtdIns(3)P is the main *in vivo* product of PI3KC2β [67]. The generation of PtdIns(3,4)P_2 by *Drosophila*, murine and human PI3KC2β was demonstrated *in vitro* in the original studies, but was not investigated later [68, 69, 72]. In addition, the *in vitro* activity of the enzyme towards PtdIns(4)P was much lower than towards PtdIns. Therefore, the clear demonstration of PtdIns(3,4)P_2 *in vivo* synthesis by PI3KC2β would be first important thing to show.

The presence of Dbl in the complex with PI3KC2β in the NIH3T3 cells over-expressing the kinase (Fig. 3-8 B), as well as the increase in Rho/Rac activity (Blajecka *et* al. 2012, manuscript accepted) in this cell line suggested that both proteins the GEF and the kinase are components of the same signaling pathway. Moreover, overexpression of PI3KC2β in mouse fibroblasts induced cell enlargement, stress fibers and ruffles formation (Fig. 3-1). A similar phenotype was observed before in NIH3T3 cells transfected with proto-Dbl or PH-Dbl domain [182, 189]. Cells were flat, elongated and slightly enlarged, the actin cytoskeleton was organized in stress fibers and membrane ruffles were visible at the cell surface [182, 189]. These results indicate that PI3KC2β and Dbl might be implicated in the same pathway controling the same cellular responses, what supports the fact that PI3KC2β may indirectly influence the GEF's activity. The reason why this could not be detected in the GEF activity assay may arise from the fact that Dbl may be more active in the cell, but not

after immunoprecipitation, during which part(s) of the complex may be lost. It is not unlikely, since isolating multiprotein complexes from cells can be problematic, for instance isolation of huge mTOR complexes [197] (Prof. Loewith, personal communication). Moreover, another possibility exists that in order to see PI3KC2β-dependent Dbl activation, cells would need to be stimulated with ligands. Stimulaton of NIH3T3 cells over-expressing PI3KC2β did not increase complex formation with endogenous Dbl (Fig. 3-8 C). However, maybe it would be needed for Dbl activation. Preliminary results from our lab showed increased Rac1 and RhoA activity upon respective stimulation with PDGF or LPA in NIH3T3-C2β-WT cells in comparison to NIH3T3-C2β-DN stable cell lines (not shown; Blajecka *et* al. 2012, manuscript accepted). It is possible that, to see the difference in PI3KC2β-WT or –DN-dependent Dbl activation, stimulation with ligands would be required. Unfortunately, there is not much data available yet concerning proto-Dbl response to external stimuli. One report describes Dbl tyrosine phosphorylation and resulting RhoGTPases activation upon EGF stimulation [188]. Possible involvement of other ligands in releasing Dbl inactive conformation awaits further examination.

Moreover, some other studies, which used NIH3T3 cells stably transfected with proto-Dbl, showed PI3K-dependent and proto-Dbl-mediated increase in Rac activation, membrane ruffles formation and cell motility [198]. Inhibition of PI3Ks with LY294002 caused enhanced proto-Dbl translocation to the membrane colocalizing with the ruffling areas, and increased GTPase activation. However, influence of the PI3K on the proto-Dbl activity *in vitro* was not shown. In the light of our results demonstrating lack of direct PI3KC2β-mediated Dbl activation, these resuts can suggest indirect involvement of PI3KC2β in the activation of the cytoskeletal rearrangements in NIH3T3 through Dbl-dependent mechanism. Whether this mechanism involves PI3KC2β-mediated generation of phosphorylated lipids in the membrane would need to be investigated.

Last but not least, the lack of an effect of PI3KC2β in the activation of Dbl GEF activity *in vitro* suggests that other molecules need to be present in the cells, which mediate the interaction of the class II PI3K with Dbl and the GEF activation, resulting in the observed phenotypes in the NIH3T3 cells. The identity of these putative additional binding partners involved in the Dbl/PI3KC2β complex is briefly discussed below.

Additional putative binding partners of Dbl/PI3KC2β complex – variations on the theme

One of the PI3KC2β binding partners, which could play a role in Dbl GEF activation, is the Grb2 adaptor molecule. It was previously shown to recruit PI3KC2β to the EGFR in the plasma membrane in both basal and EGF-stimulated conditions [94]. Moreover, Grb2 was

also reported to be crucial for the EGF-dependent Dbl phosphorylation by non-receptor tyrosine kinase ACK1 (activated Cdc42Hs-associated kinase), which was co-immunoprecipitated with Dbl [184, 188]. ACK1 is a widely expressed protein, which belongs to the focal adhesion family of kinases. Through its proline-rich motifs ACK1 is recruited to the membrane via N-terminal SH3 domain of Grb2 adaptor protein and it is activated downstream of the EGFR and PDGFR [184, 188, 199]. Upon activation, it further phosphorylates Dbl, which increases its GEF activity torward Cdc42 and Rho [184]. These data taken together with my observations that Grb2 is present in the Dbl/PI3KC2β complex in the NIH3T3 and HEK293 cells support the model that PI3KC2β builds a complex consisting of Grb2, ACK1 and Dbl. Furthermore, this complex may be important for Rho GTPases activation in serum-supplemented conditions and upon EGF or PDGF stimulation. Upon EGF stimulation PI3KC2β is recruited to the plasma membrane via Grb2, which recruits also ACK1 complexed with an active RhoGTPase. ACK1 was in fact shown to phosphorylate Dbl only when complexed with Grb2 and Cdc42-GTP [188]. Being in close proximity to GEF, ACK1 could therefore bind to Dbl and phosphorylate it leading to signaling cascade activation consisting of Cdc42, Rac and Rho that regulates stress fibers and membrane ruffles formation in fibroblasts [200]. This model seems to be consistent with my results, which showed Grb2 interaction with Dbl in the HEK293 cells pull-down (Fig. 3-4 B), although the association was not direct, according to the *in vitro* binding assay (Fig. 3-6 A). It could therefore mean that Grb2 binds to ptoto-Dbl through ACK1 non-receptor tyrosine kinase and presence of this protein is necessary for the GEF activation. A schematic model of PI3KC2β/Dbl complex formation and its activation mediated by Grb2/Ack1-dependent interactions is illustrated on Fig. 3-10. However, one has to be aware that the experiments with EGF and PDGF stumulation did not increase PI3KC2β/Dbl association, suggesting constitutive complex assembly (Fig. 3-8 C). Therefore, further investigations of the complex formation in the NIH3T3 cells co-transfected with PI3KC2β and ACK1 would be required to verify this model. In the same conditions, Dbl phosphorylation status and its activity could be studied, as well as the role of PI3KC2β in activation of the Grb2/ACK1/RhoGTPase complex.

As indicated already in the previous section, due to multi-domain structure, activation of Dbl is possibly dependent on different molecular mechanisms precisely controlled in the spatio-temporal manner. Therefore, involvement of other factors cannot be ruled out. For instance in NIH3T3 cells, Dbl binds also to the active α-subunit of the heterotrimeric G protein G13, resulting in the activation of RhoA and cytoskeletal rearrangements [186]. Proto-Dbl may be activated by Gα13 through a mechanism which involves an association with ezrin, an ezrin-radixin-moesin (ERM) family protein, which links the plasma membrane to the actin cytoskeleton [187]. When Dbl is activated, it translocates to the membrane.

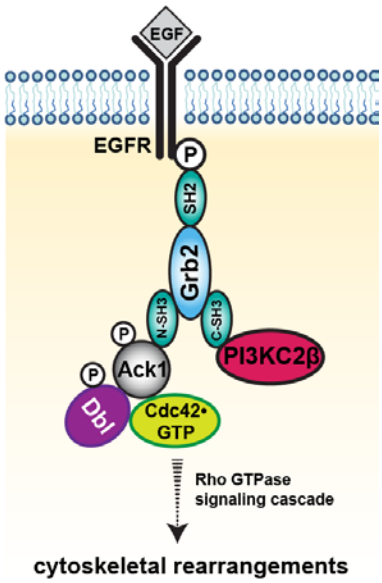

Figure 3-10. Hypothetical model of PI3KC2β/Dbl complex formation and activation. Upon EGF stimulation PI3KC2β is recruited to the plasma membrane via C-terminal SH3 Grb2 domain. Through its N-terminal SH3 domain Grb2 binds ACK1, which become activated upon association to GTP-bound Cdc42. Being in close proximity to GEF, ACK1 binds to Dbl and phosphorylates it leading to Rho GTPase signaling cascade activation that controls cytoskeletal remodeling.

Treatment of cells with LPA induced translocation of proto-Dbl to the plasma membrane, which in turn caused changes in the cell morphology, which were characterized by increased membrane ruffling and lamellipodia formation, as well as enlargement of the cell body [187]. However, when Dbl binds to the G*βγ* subunits of heterotrimeric G protein with its N-terminal sequence, its GEF activity remains unchanged [185]. G*βγ* can directly bind to the N-terminal 53-99 residues of proto-Dbl but it does not increase Dbl GEF activity towards RhoA and Cdc42. Therefore additional factors may be required for full activation of Dbl [185]. EGFR/PI3KC2β- and Ack1-dependent signals may cooperate with G*βγ* to trigger Dbl activation, because numerous types of receptors are likely to be activated simultaneously *in vivo* [201].

Dbl is also known to interact with membrane-cytoskeleton linkers, which control actin-based cellular functions such as cell migration or adhesion [202]. In addition to other GEFs, it has been shown recently to associate with an active myosin II (MII) assembled with actin filaments in NIH3T3 cells [203]. Non-muscle MII controls RhoGTPases activity and in turn stress fibers, cell protrusion and focal complex formation in migrating cells. The interaction of MII with Dbl family GEFs blocks their activity toward GTPases. However, PDGF-induced inhibition of MII activity leads to the release of βPIX GEF, and in turn promotes transient increase of RhoGTPases activity [203]. It would be therefore interesting to investigate whether PI3KC2β/Dbl complex formation in NIH3T3 cells is somehow related to myosin II-dependent regulation of GTPases and cytoskeleton rearrangements. The class II PI3KC2α can increase contractility of vascular smooth muscle cells by stimulation of MII interaction with actin filaments via Ca^{2+}-dependent Rho signalling pathway [119]. Further studies are needed to verify whether PI3KC2β would be able to control RhoGTPases activity by MII mode of action.

Potential role of PI3KC2B/Dbl interaction in neuroblastoma

There have been many interesting reports published in recent years about the class II PI3KC2β involvement in human cancer. They emphasize an important role of the kinase in cytoskeletal rearrangements resulting in increased cancer cell migration, invasion and metastasis, but also its function in resistance to chemotherapeutics [83, 103, 139, 141]. Amplification or up-regulation of the PI3KC2β gene and protein have been shown in numerous types of human cancers not excluding neuroendocrine tumors, which arise from endocrine and nervous systems, such as small cell lung cancer and neuroblastoma [129]. Various PI3KC2β expression levels in different types of tumors might result from its ubiquitous expression and possible involvement in some general molecular mechanisms and responses, present in every cell, independently of the tissue and organs. Dbl proto-oncogene expression on the other hand, has been confined to the specific tissues of neuroendocrine and neuroectodermal origin (gonads and central or peripheral nervous system), as well as to some cancers, which derive form that tissues [170, 191-193]. It can therefore play more specialized functions than PI3KC2β. Even within the tumors of the same origin it was not equally expressed suggesting its unique function. Although in some studies proto-Dbl mRNA expression was not found in neuroblastoma specimens and cell lines [191, 192], in other research oncogenic Dbl was detected in the IMR32 neuroblastoma cell line [193], suggesting cell line specific expression. Similarly, in my results I could show proto-Dbl expression in IMR5 neuroblastoma cells, which also displayed relatively high PI3KC2β expression and further formed a complex with endogenous Dbl (Fig. 3-3 B). Up-regulation of PI3KC2β expression in IMR5 cell line was also shown recently by Russo and O'Bryan [114]. Moreover, a strong functional dependence of PI3KC2β and intersectin 1 (INTS1) scaffolding molecule has been observed. Over-expression of PI3KC2β in the IMR5 cell line, which was intersectin 1 (INTS1)-silenced, rescued anchorage-independent growth of these cells, confirming the fact that PI3KC2β is essential for neuroblastoma tumorigenesis [114]. A physical interaction of the PI3KC2β N-terminus (proline-rich motifs) with INTS SH3 domain was described before and it was shown to be important for increase of PI3KC2β activity and Akt-dependent regulation of neuron cells survival [104]. My results demonstrate that PI3KC2β interacts with Dbl in IMR5 neuroblastoma cells suggesting potential role of the complex in this cancer progression. It would be interesting to investigate whether there is a similar functional interdependence between the kinase and the GEF as in the case of PI3KC2β and INTS in neuroblastoma cells. Beside its multi-domain scaffolding role, INTS can also possess guanine exchange factor activity toward Cdc42, which is dependent on the splice variant [114]. A direct association of PI3KC2β and Dbl was however not found in the *in vitro* binding assay (Fig. 3-6 A), as well as increased Dbl GEF activity when co-transfected with PI3KC2β (Fig. 3-9). It is therefore possible that there is no strong or straightforward interedependence

between the two proteins like it was shown for PI3KC2β and INST. Definitely, more studies would be needed to reveal potential role and functions of PI3KC2β/Dbl complex formation in neuroblastoma.

PI3KC2β and onco-Dbl – is there a common pathway in cancer?

An interesting result was obtained when PI3KC2β was immunoprecipitated from A-431 epidermoid carcinoma cells over-expressing the kinase and incubated with lysates of COS-1 cells transfected with Dbl domains. In contrast to pull-downs performed in the non-cancerous COS-1 cell line, PI3KC2β bound only to onco-Dbl mutant, but not to spectrin- and PH- domains (Fig. 3-7). Although these two experiments cannot be fully compared due to a slightly different experimental setting, they suggest that in some specific conditions, PI3KC2β has a potential to bind not only prototype Dbl, but also its oncogenic counterpart. The hypothesis that PI3KC2β immunoprecipitated from A-431-C2β cells function in the complex, which preferentially interact with onco-Dbl than with proto-Dbl domains might be supported by the fact that regulation of onco-Dbl in many different aspects seems to be very different than that of proto-Dbl [182]. Major discrepancies concern proteins localization, stability and transforming potential and they all arise from the differences in the domain structure of prototype and oncogenic Dbl. The presence in proto-Dbl of the N-terminal domain, which interacts with C-terminal PH module, limits membrane localization of the protein and completely prevents its cytoskeletal association. The N terminus of proto-Dbl dictates perinuclear and cytosolic distribution pattern of the protein, whilst the onco-Dbl localize to the plasma membrane and actin cytoskeleton components such as actin stress fibers [178, 182]. The PH domain of onco-Dbl is in fact fully exposed and due to that is able to interact with cytoskeleton proteins in the absence of lipid binding capacity [182]. It can therefore partially explain why PI3KC2β immunoprecipitated from A-431-C2β cells bound preferentially to onco-Dbl, but not to proto-Dbl domains (Fig. 3-7 A). To some extent PI3KC2β localized to cell protrusions and ruffling regions of the plasma membrane, where Eps8 and Abi1 were also localized [103]. Since these proteins were isolated together from A-431-C2β cells in one PI3KC2β-Eps8/Abi1/Sos1 complex [103], it is likely that they were present also in the PI3KC2β immunoprecipitate. It is therefore reasonable to assume that in the pull-down with COS-1 cells transfected with Dbl domains, they could become a target for onco-Dbl rather than N-terminus and spectrin domains, which do not bind cytoskeletal proteins [178]. In this context however it is difficult to explain why PI3KC2β did not bind PH-Dbl domain, since it was shown before to colocalize with actin stress fibers and it determines onco-Dbl distribution pattern. Therefore additional possibility exists, which includes a contribution from

the EGFR. A-431 cells over-express the EGFR and PI3KC2β binds to EGFR through Grb2 [94].

Onco-Dbl was not frequently detected in primary cancer specimens or cell lines. It was mostly the proto-Dbl transcript of 5 kb size, which was found over-expressed in neuroectodermal tumors [191, 192]. However, the 2.8 kb N-terminal-truncated RNA form of Dbl was detected in a set of primary tumors, nude mice xenografts and cell lines of variety of childhood tumors, such as Ewing's sarcoma, PNET, NB, rhabdomyosarcoma, retinoblastoma, and some of the non-neural tumors, such as synovial sarcoma and osteosarcoma [193]. It is still not clear how Dbl oncogenic mutation can be triggered in cancer. At the time of onco-Dbl identification, it was not present in the oncogenic form in the DNA of the original NPDL or human diffuse B cell lymphoma, but it was detected in the DNA after transfection into NIH3T3 cells, suggesting that oncogenic truncation mutation might be induced by the process of gene transfer *in vitro* or might be present *in vivo* in only a minority of tumor cells [166]. However, since onco-Dbl was detected in some tumors, where PI3KC2β was found over-expressed [129], and since I have found an interaction between these two proteins, we cannot exclude that they may play a potential role also in the onco-Dbl-dependent tumorigenesis.

3.1.6. Conclusions and outlook

The aim of this project was to investigate the mechanism of PI3KC2β/Dbl interaction and the role it plays in cytoskeletal remodelling in mouse fibroblasts. An association between endogenous PI3KC2β and Dbl in NIH3T3 and neuroblastoma cancer cells IMR5 was observed. However, PI3K kinase activity did not seem to be required for the complex assembly. Growth factors stimulation did not enhance the interaction, suggesting a constitutive complex formation. The PI3KC2β/Dbl binding was mediated by the N-terminal regulatory region of the kinase and Dbl spectrin- and PH- GEF domains. These interactions resembled an association of proto-Dbl with the Hsc70-Hsp90 chaperone complex, but did not appear to be direct, suggesting the involvement of additional binding partners mediating PI3KC2β/Dbl association and indicating a more complicated mechanism of PI3KC2β-dependent Dbl regulation. These results were supported by the lack of GEF activity of Dbl toward Rho GTPase in the *in vitro* GEF activity assay. No difference in Dbl activity was observed upon its isolation from HEK293 cells transfected with PI3KC2β wild-type and dominant-negative (kinase-dead) form. Therefore, the possibility exists that some additional factors are needed to induce Dbl activation in a PI3KC2β-dependent manner. One of the candidates could be the ACK1 non-receptor tyrosine kinase, which was shown to phosphorylate and thus activate Dbl upon EGF stimulation in a Grb2- and Rho GTPase-mediated manner. Consequently, the phosphorylation status of Dbl, as well as the PI3KC2β/Dbl complex assembly could be investigated upon co-transfection of the ACK1 and PI3KC2β-WT and –DN in mouse fibroblasts.

The role of PI3KC2β in the complex with Dbl remains not completely understood. It is not clear whether it plays kinase activity-independent scaffolding role or whether its PI3K function is essential for indirect Dbl activation in the multi-protein complex. Taking into consideration the complicated mechanism of the GEF regulation it is likely that PI3KC2β lipid substrates or products in the plasma membrane may be important for modulation of Dbl activity and function. To become fully active GEFs require combination of different molecular events and multiple signals, which includes also translocation to the plasma membrane or protein-protein interactions. It is possible that, similarly to PI3KC2α, the PI3KC2β isoform activates RhoGTPases in NIH3T3 cells in concert with Dbl and actin filament-associated proteins such as MII. These and other aspects of the PI3KC2β-dependent machinery in NIH3T3 cells such as direct evidence that the PI3KC2β/Dbl complex is needed for the observed phenotype would be worth studying. It would be important to clarify whether PI3KC2β positively affects RhoA activation and if the Dbl binding is of any function. Investigating PI3KC2β/Dbl complex assembly in human cancer cells, such as neuroblastoma cells could be another exciting area of reaserch.

3.2. Identification and Functional Characterization of PI3KC2β N-terminus Tyrosine Phosphorylation Sites (Project II)

3.2.1. Summary

To date the posttranslational modifications role of class II PI3Ks in the enzymes' regulation and functions in different cellular responses has not been extensively studied. PI3KC2β tyrosine phosphorylation has been found in few cancerous and non-cancerous cell lines upon RTKs stimulation. In order to investigate PI3KC2β regulation by tyrosine phosphorylation, as well as the functional relevance of PI3KC2β tyrosine phosphorylation sites, we identified by mass spectrometry four new tyrosine phosphorylation sites in PI3KC2β isolated from human cancer cell lines. Y68, Y127, Y228 and Y1541 appeared to be consensus sites among different vertebrate species indicating a conservation of their function. Mutation of tyrosine 127 and 228 to phenylalanine in the N-terminal regulatory region of the PI3KC2β wild-type induced increased cell proliferation and cell-cell adhesions formation in HT-29 colon cancer cells stably transfected wih the double mutant. Similar results were obtained for PI3KC2β kinase-dead form of the enzyme, indicating kinase-dependent mechanism of regulation. In contrast, PI3KC2β-WT transfection in HT-29 cells had no impact on cell proliferation and adherens junction formation. Furthermore, activation of Akt increased upon EGF stimulation of HT-29 cells over-expressing PI3KC2β Y127/228F mutant in comparison to cells expressing PI3KC2β-WT. These results suggest that phosphorylation of Y127 and Y228 in PI3KC2β-WT may negatively regulate PI3KC2β function and downstream signaling through generation of phosphorylated lipids in the plasma membrane. Taken together, our findings reveal for the first time that PI3KC2β tyrosine phosphorylation of N-terminal regulatory domain contributes to the control of cell proliferation and the formation of cell-cell junctions in human cancer cells.

3.2.2. Introduction

PI3KC2β belongs to the class II family of conserved phosphoinositide 3-kinases, which was shown to be essential for cell migration/adhesion processes, protection against anoikis and cell proliferation downstream of RTKs and GPCRs [83, 84, 103]. Association with RTKs such us EGFR, PDGF, c-Kit, c-Met and IGF-IR upon ligand stimulation has been shown to be necessary for the enzyme activation in a variety of cell line models [77, 99]. Tyrosine phosphorylation of PI3KC2β has been observed, but a detailed functional investigation of site-specific tyrosine phosphorylations has never been performed. Minimal knowledge about the functional context of the other post-translational modifications of PI3KC2β comes from open access data bases, which gather the information from a number of high-throughput studies. In one of these studies PI3KC2β S155 phosphorylation has been found in the set of proteins activated during mitosis, whose phosphorylation is cell cycle-regulated [204]. In this study, S155 phosphorylation was observed in the M-phase, whereas increased abundance of the protein was detected in G1 phase.

Identification of the new tyrosine phosporylation sites in PI3KC2β

In order to understand potential role of tyrosine phosphorylation in the regulation of PI3KC2β functions, we have mapped *in vivo* tyrosine phosphorylation sites within PI3KC2β. We employed immunoaffinity purification with P-Tyr-100 antibody followed by tandem mass spectrometry (LC-MS/MS) analysis of enriched phospho-tyrosine peptides, from different human cancer cell lines, treated with or without pervanadate/calyculin, 10% serum or EGF. PhosphoScan/PTMScan has been performed by Cell Signalling Technology (CTS) (www.cellsignal.com). Since 10% serum and EGF stimulation are commonly used to induce protein phosphorylation, pervanadate and calyculin were respectively used as tyrosine and serine/threonine protein phosphatase activity inhibitors to artificially enhance levels of protein phosphorylation in the studied cell lines (for detailed list of cell lines and treatments check Table 3-1). This functional analysis revealed four novel tyrosine phosphorylation sites within the PI3KC2β sequence (Table 3-1). Three of them (Y68, Y127, Y228) were clustered in the N-terminal region of the enzyme while the fourth (Y1541) was located in the C-terminal C2 domain. Fig. 3-11 A and B respectively show the location of newly detected tyrosine phosphorylation sites in the sequence and domain structure of PI3KC2β.

Table 3-1. PI3KC2β phospho-tyrosine sites (pY) identified in human cancer cell lines

PI3KC2β pY	Site sequence	High quality spectra
Y68	DEPGVDF**Y**SKPAGRR	Jurkat||pervanadate; Jurkat||pervanadate|calyculin; SEM; SEM||10% serum
Y127	KGSLSGD**Y**LYIFDGS	HT29||EGF; KG-1; SUP-M2
Y228	RLLGSVD**Y**DGINDAI	H1650||EGF|Iressa); H196||EGF; H226||EGF; H460||EGF; HT29||Serum starved|EGF; JPV-CONT||calyculin; Jurkat||anti-CD3|anti-mouse Ig|anti-CD28; Jurkat||calyculin|pervanadate; Jurkat||pervanadate; Jurkat||pervanadate|calyculin; LNCaP||EGF; MO-91; SEM; SEM||10% serum; SUP-M2
Y1541	DGNDPDP**Y**VKIYLLP	MO-91; SNU-5

Information about PI3KC2β tyrosine phoshorylation can be found on www.phosphosite.org.

The N-terminal phosphorylation of Y68 seems to be important in human T-cell and B-cell leukemia cells (Jurkat and SEM, respectively), since it was the only type of cancer where it was identified after pervanadate/calyculin or 10% serum treatment (Table 3-1). The Y127 phospho-site was also observed in leukemic cells (KG-1), although without any treatment, as well as in the SUP-M2 lymphoma cell line. On the other hand, EGF stimulation of HT-29 colorectal carcinoma cells resulted in phosphorylation of the Y127 residue, as well as phosphorylation of the Y228 site, which was also found in a number of lung cancer cell lines (H1650, H196, H226, H460) or prostate cancer cells (LNCap) upon EGF stimulation. As in the case of Y68, phosphorylation of Y228 was further observed in Jurkat and SEM cells after pervanadate/calyculin or 10% serum treatment or in MO-91 leukemia or SUP-M2 lymphoma cell line in the absence of any treatment. Phosphorylation of the C-terminal Y1541 site was detected in MO-91 and SNU-5 gastric cancer cells (Table 3-1). The obtainded results indicate that among the identified PI3KC2β phospho-tyrosine sites, Y228 is the most frequently phosphorylated in the investigated human cancer cells, suggesting a possibly important role for PI3KC2β activation and function.

A

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MSSTQGNGEH WKSLESVGIS RKELAMAEAL QMEYDALSRL RHDKEENRAK QNADPSLISW 60
DEPGVDFYSK PAGRRTDLKL LRGLSGSDPT LNYNSLSPQE GPPNHSTSQG PQPGSDPWPK 120
GSLSGDYLYI FDGSDGGVSS SPGPGDIEGS CKKLSPPPLP PRASIWDTPP LPPRKGSPSS 180
SKISQPSDIN TFSLVEQLPG KLLEHRILEE EEVLGGGGQG RLLGSVDYDG INDAITRLNL 240
KSTYDAEMLR DATRGWKEGR GPLDFSKDTS GKPVARSKTM PPQVPPRTYA SRYGNRKNAT 300
PGKNRRISAA PVGSRPHTVA NGHELFEVSE ERDEEVAAFC HMLDILRSGS DIQDYFLTGY 360
VWSAVTPSPE HLGDEVNLKV TVLCDRLQEA LTFTCNCSST VDLLIYQTLC YTHDDLRNVD 420
VGDFVLKPCG LEEFLQNKHA LGSHEYIQYC RKFDIDIRLQ LMEQKVVRSD LARTVNDDQS 480
PSTLNYLVHL QERPVKQTIS RQALSLLFDT YHNEVDAFLL ADGDFPLKAD RVVQSVKAIC 540
NALAAVETPE ITSALNQLPP CPSRMQPKIQ KDPSVLAVRE NREKVVEALT AAILDLVELY 600
CNTFNADFQT AVPGSRKHDL VQEACHFARS LAFTVYATHR IPIIWATSYE DFYLSCSLSH 660
GGKELCSPLQ TRRAHFSKYL FHLIVWDQQI CFPVQVNRLP RETLLCATLY ALPIPPPGSS 720
SEANKQRRVP EALGWVTTPL FNFRQVLTCG RKLLGLWPAT QENPSARWSA PNFHQPDSVI 780
LQIDFPTSAF DIKFTSPPGD KFSPRYEFGS LREEDQRKLK DIMQKESLYW LTDADKKRLW 840
EKRYYCHSEV SSLPLVLASA PSWEWACLPD IYVLLKQWTH MNHQDALGLL HATFPDQEVR 900
RMAVQWIGSL SDAELLDYLP QLVQALKYEC YLDSPLVRFL LKRAVSDLRV THYFFWLLKD 960
GLKDSQFSIR YQYLLAALLC CCGKGLREEF NRQCWLVNAL AKLAQQVREA APSARQGILR 1020
TGLEEVKQFF ALNGSCRLPL SPSLLVKGIV PRDCSYFNSN AVPLKLSFQN VDPLGENIRV 1080
IFKCGDDLRQ DMLTLQMIRI MSKIWVQEGL DMRMVIFRCF STGRGRGMVE MIPNAETLRK 1140
IQVEHGVTGS FKDRPLADWL QKHNPGEDEY EKAVENFIYS CAGCCVATYV LGICDRHNDN 1200
IMLKTTGHMF HIDFGRFLGH AQMFGNIKRD RAPFVFTSDM AYVINGGDKP SSRFHDFVDL 1260
CCQAYNLIRK HTHLFLNLLG LMLSCGIPEL SDLEDLKYVY DALRPQDTEA NATTYFTRLI 1320
ESSLGSVATK LNFFIHNLAQ MKFTGSDDRL TLSFASRTHT LKSSGRISDV FLCRHEKIFH 1380
PNKGYIYVVK VMRENTHEAT YIQRTFEEFQ ELHNKLRLLF PSSHLPSFPS RFVIGRSRGE 1440
AVAERRREEL NGYIWHLIHA PPEVAECDLV YTFFHPLPRD EKAMGTSPAP KSSDGTWARP 1500
VGKVGGEVKL SISYKNNKLF IMVMHIRGLQ LLQDGNDPDP YVKIYLLPDP QKTTKRKTKV 1560
ARKTCNPTYN EMLVYDGIPK GDLQQRELQL SVLSEQGFWE NVLLGEVNIR LRELDLAQEK 1620
TGWFALGSRS HGTL                                                  1634
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B

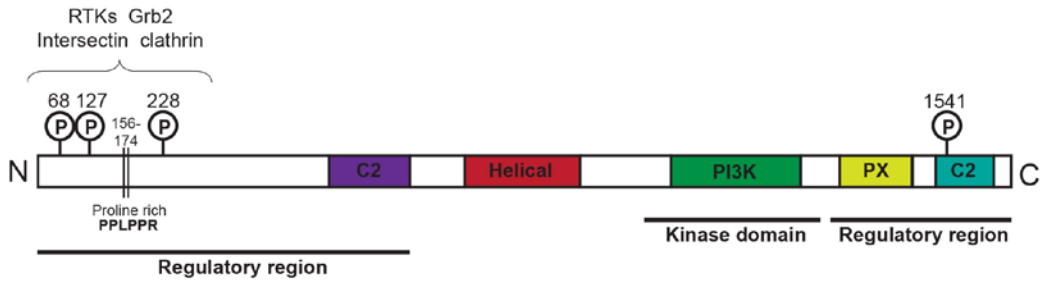

Figure 3-11. Distribution of newly identified pY sites in PI3KC2β amino acid sequence and domain structure. (A) Localization of Y68, Y127, Y228 and Y1541 phosphorylation sites in PI3KC2β amino acid sequence retrieved from GenBank (accession no. NM_002646). pY residues are underlined and indicated in red, proline-rich regions are underlined in black. (B) Domain structure of PI3KC2β with indicated positions of defined tyrosine phosphorylation sites and RTKs, Grb2, intersectin and clathrin binding regulatory region. PI3K – catalytical domain, PX – Phox domain.

PIK3C2B gene homology has been found among different vertebrate species from zebrafish through chicken, cow, mouse, rat, dog, monkey, chimpanzee and human (source: www.ncbi.nlm.nih.gov/sites/homologene/20582). We were therefore interested whether our newly identified phospho-tyrosine sites within human PI3KC2β are conserved throughout evolution and thus functionally important. Indeed, multiple sequence alignment between *H.sapiens* and other vertebrate species revealed that all Y68, Y127, Y228 and Y1541 are consensus sites, indicating conservation of function in this region (Fig. 3-12). Y228 and Y1541 are present in all listed species, whereas both Y68 and Y127 are absent in zebrafish, and Y68 is further absent in chicken.

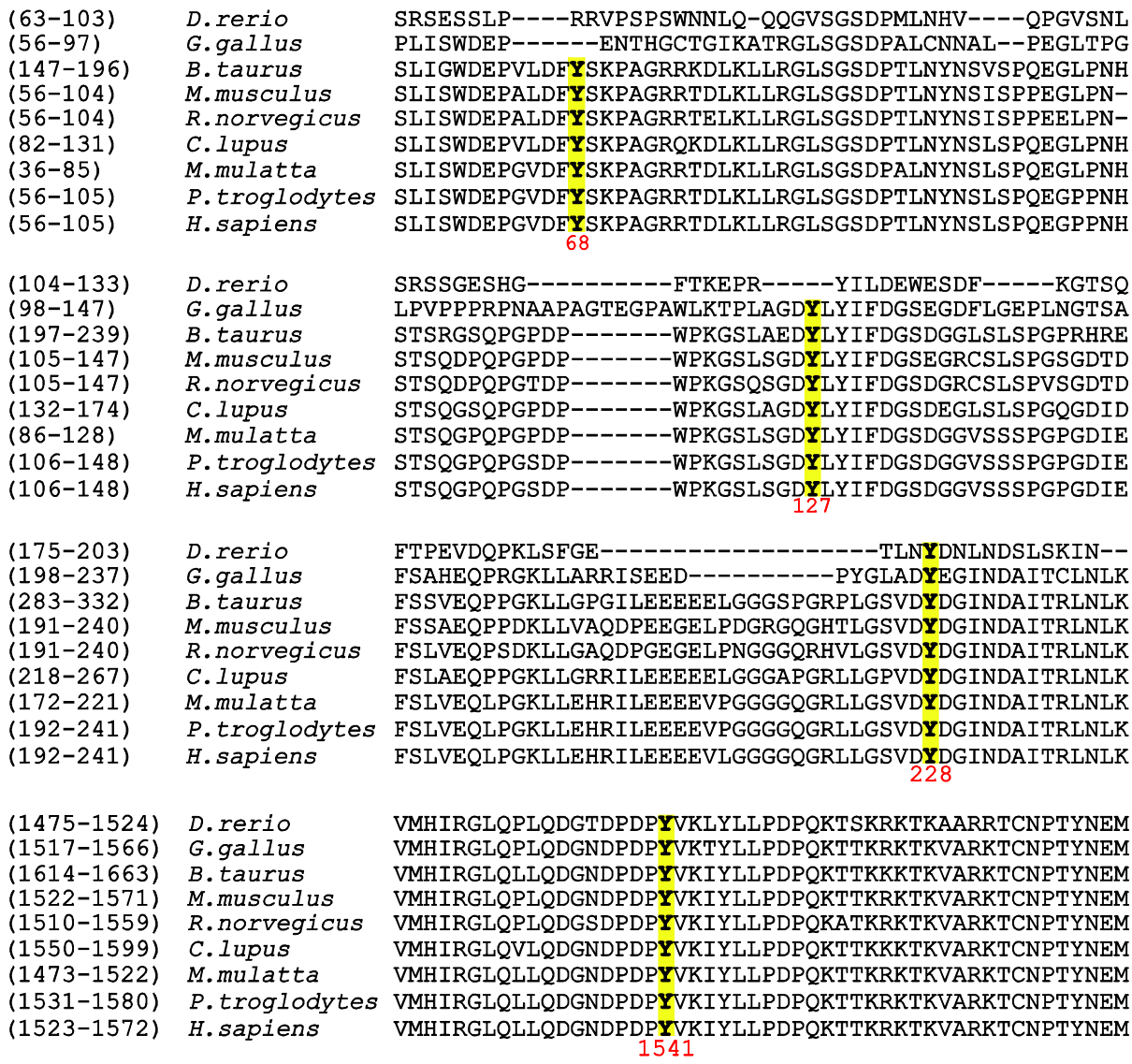

Figure 3-12. Conservation of the newly identified PI3KC2β pY sites between different vertebrate species. The sequence of human PI3KC2β (GenBank accession no. NP_002637.3) was aligned with the sequences from zebrafish *(D.rerio)* (GenBank accession no. XP_003199535.1), chicken *(G.gallus)* (GenBank accession no. XP_417956.3), cow *(B. taurus)* (GenBank accession no. XP_002693928.2), mouse *(M.musculus)* (GenBank accession no. NP_001092746.2), rat *(R.norvegicus)* (GenBank accession no. NP_001099421.1), dog *(C. lupus*) (GenBank accession no. XP_536097.3), rhesus monkey *(M.mulatta)* (GenBank accession no. XP_002802010.1) and chimpanzee *(P.troglodytes)* (GenBank accession no. XP_514126.3) using the NCBI HomoloGene multiple alignment tool (www.ncbi.nlm.nih.gov/sites/homologene/20582). pY consensus sites are marked in bold and highlighted in yellow. The numbering of Y68, Y127, Y228 and Y1541 residues is based on the sequence of human PI3KC2β and may slightly vary for other species.

Experimental strategy

Y228 was the most frequently phosphorylated residue within PI3KC2β among the identified phospho-tyrosine sites in human cancer cells. Additionally, it appeared to be a consensus site conserved throughout evolution among different vertebrates, from zebrafish to human, suggesting a possible functional importance. Therefore, as an *in vitro* model to study Y228 functional relevance, we first selected the HT-29 human intestinal cancer cells, as one of the cell lines where this phospho-site was detected upon EGF stimulation. Similarly, Y127 phosphorylation was found in these cells, which provided an option for studying both tyrosine phosphorylation sites simultaneously. HT-29 cells appeared to be a good model to work with due to simple and easy culture requirements. This cell line was established from a colon adenocarcinoma and is often used to investigate epithelial differentiation and cell polarity [205-207]. However, PI3KC2β was shown not to be essential for differentiation *in vivo* at least in the epidermis [123]. PI3KC2β's role in HT-29 cells might be therefore very different. According to previous investigations of PI3KC2β functions in human epidermoid carcinoma A-431 cells, PI3KC2β was associated to pro-survival and pro-migratory responses, as well as to increased cell proliferation [103]. To investigate the functional relevance of 127 and 228 phospho-tyrosine sites we decided to generate stable clones of HT-29 cells over-expressing PI3KC2β single (Y127F, Y228F) and double (Y127/228F) phospho-tyrosine site mutants. Futhermore, functional responses of the newly generated HT-29 cell lines over-expressing PI3KC2β mutants and wild-type as control were compared in terms of cell proliferation, cell morphology and activation of downstream signalling pathways.

3.2.3. Results

Generation of phospho-tyrosine (pY) mutants and their validation

To elucidate the functional relevance of tyrosine 127 and 228 phosphorylation sites identified in PI3KC2β, we generated tyrosine to phenylalanine (Y/F) mutants, which cannot be phosphorylated anymore at the identified tyrosine sites. Single (Y127F, Y228F) and double (Y127/228F) Y/F mutants in the full-length enzyme were produced by PCR-based site-directed mutagenesis followed by mutant constructs verification by DNA sequencing (Top Gene Technologies, Inc., Canada). As a basis for site-directed mutagenesis, the previously reported construct of pcDNA3 vector containing the N-terminal Myc epitope tag (MEQKLISEEDL) PI3KC2β wild-type cDNA sequence was used [72]. An N-terminal Myc epitope tag was therefore included in the pY mutants' cDNAs to facilitate further isolation of the mutated proteins from transfected cells by immunoprecipitation. In order to verify the accuracy of the constructs we additionally performed triple restriction digestion with EcoRI, XhoI and RsrII enzymes.

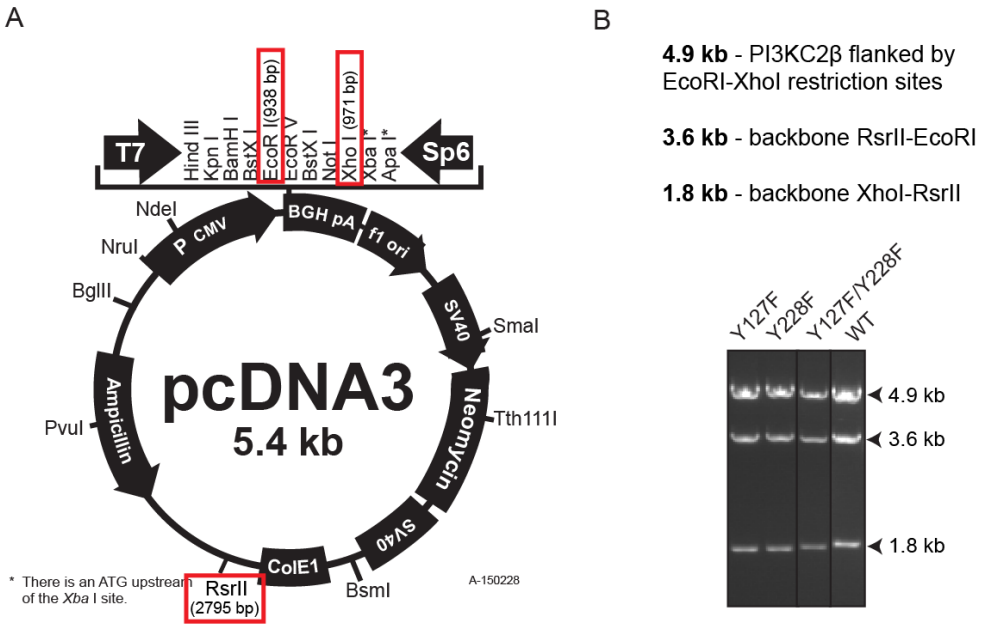

Figure 3-13. Verification of PI3KC2β Y/F mutants constructs in triple restriction digestion. (A) Schematic representation of pcDNA3 backbone vector (Invitrogen) used for PI3KC2β wild-type and pY mutants generation. EcoRI, XhoI and RsrII restriction sites are indicated in red frames. (B) Predicted sizes of potential DNA fragments obtained in the triple restriction digestion and the agarose gel presenting the results for Y127F, Y228F single and Y127/228F double mutants, and PI3KC2β wild-type (WT) control.

Fig.3-13 A shows the exact location of the particular restriction sites in the pcDNA3 vector, whereas Figure 3-13 B presents the predicted sizes of potential DNA fragments obtained as a result of enzymatic digestion, and the actual results after electrophoretic separation on agarose gel. The PI3KC2β wild-type plasmid was included as a control. Correct sizes of DNA fragments were observed for all plasmids, thus confirming the accuracy of generated mutant constructs.

Differential expression of PI3KC2β Y/F mutants at the mRNA and protein level

In order to characterize PI3KC2β Y/F mutants behaviour in the mammalian cell systems I transfected the Y127F and Y228F single mutants, as well as the Y127F/Y228F double mutant, PI3KC2β wild-type (WT) and pcDNA3 empty vector as controls into HEK293 cells. Their expression at the mRNA and protein level was further investigated with a qPCR and western blot, respectively. Results showed differential expression levels of mRNA (Fig. 3-14 A), which correlated with the expression of the mutated proteins (Fig. 3-14 B).

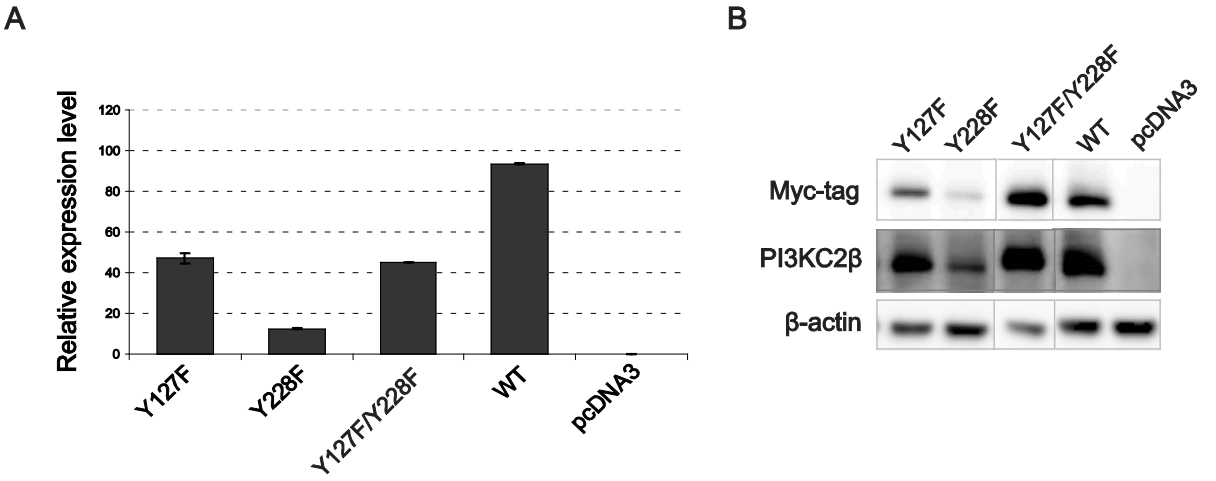

Figure 3-14. Differential expression of PI3KC2β Y/F mutants mRNA and protein in HEK293 cells. HEK293 cells were transfected with PI3KC2β Y/F mutants and analysed 24 h post-transfection for mRNA expression by real-time PCR (A) and 48 h post-transfection for the protein expression by western blot (B).

Similarly, I performed transient transfection experiments of pY mutants into HT-29 cells and mRNA and protein expression levels were evaluated. Semi-quantitative RT-PCR analysis showed quite equal expression of the mutants' transcripts in comparison to GAPDH control, detected with a use of primers specific for exogenous PI3KC2β cDNA containing Myc-tag sequence (Fig. 3-15 B). On the other hand, an endogenous PI3KC2β was also detected in the HT-29 cell line by the standard RT-PCR. Real-time PCR analysis of Y127F, Y228F,

Y127F/Y228F mutants' cDNAs revealed differential expression of transcripts detected 48 h post-transfection (Fig. 3-15 C). However, the differences between the mutants were minor, which was also observed at the protein level (Fig. 3-15 D). The transfection efficiency measured by GFP signal analysis by fluorescence microscopy was relatively high (Fig. 3-15 A).

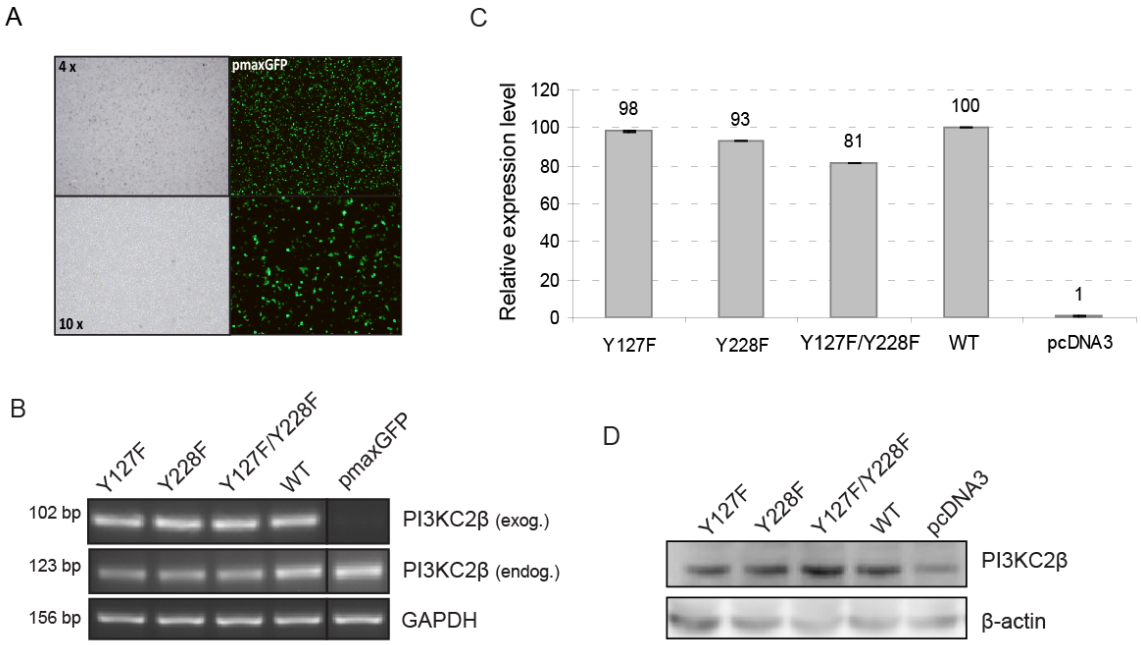

Figure 3-15. mRNA and protein expression level of PI3KC2β Y/F mutants in HT-29 cells. HT-29 cells were transfected with single (Y127F, Y228F) and double (Y127F,Y228F) PI3KC2β mutants and control vector pmaxGFP (A,B) or pcDNA (C,D). (A) Transfection efficiency visualized by GFP signal analysis with fluorescent microscope 48 h post-transfection. (B) mRNA expression level of the PI3KC2β Y/F mutants and endogenous PI3KC2β transcripts analyzed by standard RT-PCR 48 h post-transfection. (C) mRNA expression level of the PI3KC2β Y/F mutants analyzed by real-time PCR 48 h post-transfection. (D) PI3KC2β Y/F mutants protein expression level analyzed by western blot 48 h post-transfection.

Generation of stably transfected HT-29 cell lines expressing PI3KC2β Y/F mutants

For further investigation of the impact of tyrosine phosphorylation on the PI3KC2β functions and signaling, we decided to establish HT-29 cell lines stably transfected with the Y127F and Y228F single mutants and Y127/Y228F double mutant. As a control, we further generated PI3KC2β wild-type (WT), PI3KC2β kinase-dead (KD) and pcDNA3 empty vector (V) expressing HT-29 cells. Stable clones were selected in a medium containing 1mg/ml geneticin (G418). Transfection efficiency was evaluated by quantifying both mRNA and protein level by qPCR and western blot, respectively. Based on this analysis we selected two stable clones for each PI3KC2β mutants exhibiting a 3-5 fold higher expression of the gene

than HT-29 cells transfected with the empty vector control (Fig. 3-16). The clones, which expressed the highest level of PI3KC2β were further expanded and subjected for phosphorylation studies, as well as investigation of cellular responses.

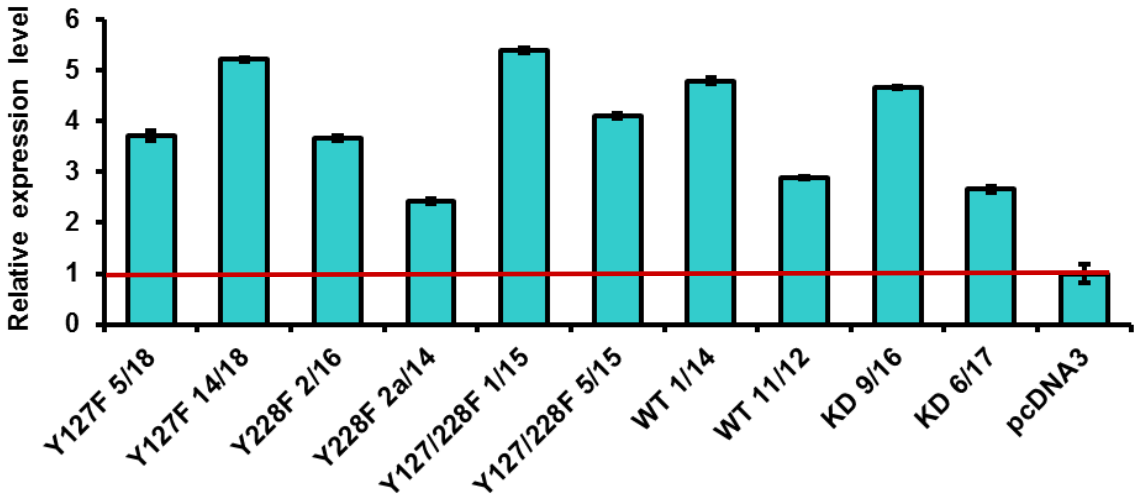

Figure 3-16. Positive clones of PI3KC2β transfectants displaying the highest mRNA expression level among all stable cell lines. HT-29 cells were transfected with PI3KC2β Y/F single and double mutants (Y127F, Y228F and Y127F,Y228F), wild-type (WT) and kinase-dead (KD) constructs, as well as pcDNA3 empty vector as control. Stably expressing clones were selecetd with G418 (1mg/ml) and expanded for further screening by real-time qPCR and western blot.

Phosphorylation of endogenous PI3KC2β in HT-29 cells

PI3KC2β was previously shown to be tyrosine phosphorylated in different cell lines such as NIH3T3, A-431, HEK293, SCLC and H-209 [77, 99]. Among the range of human cancer cell lines used for our pY screen, PI3KC2β was found to be tyrosine phosphorylated in HT-29 human colon adenocarcinoma cells. In order to confirm the mass spectrometry results, I performed immunoprecipitation of the endogenous PI3KC2β from HT-29 cells upon EGF stimulation followed by the whole protein tyrosine phosphorylation analysis. Indeed, increased tyrosine phosphorylation after stimulation with EGF in comparison to starved cells was observed (Fig. 3-17).

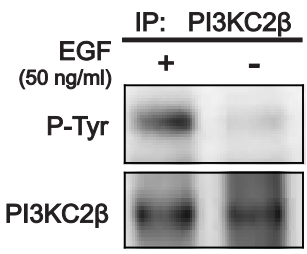

Figure 3-17. Tyrosine phosphorylation of the endogenous PI3KC2β in HT-29 cells. HT-29 cells were cultured in 10% FCS containing medium for 24 h. Next, they were serum-starved (1% FCS) over-night, and were stimulated with EGF (50 ng/ml) for 10 min. Cell lysates were equalized for protein content and subjected to immunoprecipitates prepared with anti-PI3KC2β antibody were analysed by western blot with the indicated antibodies.

Phosphorylation of the PI3KC2β Y127/228F mutant in stably transfected HT-29 cells

HT-29 cells provided us an opportunity to study Y127 and Y228 phospho-sites simultaneously under the same conditions. Therefore, in order to verify the phosphorylation status of pY PI3KC2β mutants, I first concentrated my efforts on characterizing the Y127/228F mutated protein in comparison to the PI3KC2β -WT, -KD and empty vector as controls. For that purpose, I used two HT-29 cell lines expressing the PI3KC2β Y127/228F double mutant, which originate from two separate stable clones. I starved the cells over-night in 0.5% FCS and 1 h in FCS-free medium and further stimulated them for 5 min. with EGF at the concentration of 150 ng/ml, which was used previously in HeLa cells for determining of phosphorylated peptides by mass spectrometry [208]. Under these conditions PI3KC2β Y228 phosphorylation site was identified. Immunoprecipitation with an anti-Myc-tag antibody was performed on the indicated cell lines and the samples were further subjected to tyrosine phosphorylation analysis by western blot (Fig. 3-18 A). The Myc-tag antibody was further used after stripping the membranes to determine PI3KC2β immunoprecipitation efficiency from the whole cell lysates. The results revealed double bands at the size of PI3KC2β. Of these two the upper one was assigned to PI3KC2β, as confirmed by overlap of phospho-Tyr with Myc-tag antibody signal. As shown before, different Y127F/Y228F stable clones did not express the same mRNA and protein levels (Fig. 3-16 and 3-18 B). It was therefore quite difficult to assess their tyrosine phosphorylation status. For this reason, we focused our attention on the 1/15 HT-29 stable cell line expressing Y127/228F double mutant and HT-29 cells expressing PI3KC2β-WT. As indicated in the whole cell extracts (WCEs) and Myc-tag immunoprecipitation (Fig. 3-18 A, B), the expression levels of the mutated kinase in 1/15 HT-29 cells was similar to the WT protein expression in contrast to 5/15 HT-29 stable cell line. Relative quantification of the ratio of phospho-Tyr and Myc-tag bands intensities in HT-29 1/15 cells and wild-type expressing HT-29 cell line upon immunoprecipitation revealed that PI3KC2β Y127/228F mutant is less phosphorylated than the wild-type enzyme (Fig. 3-18 A, bottom panel). This effect was observed in both, starved and EGF stimulated cells, but interestingly under FCS-free conditions the difference was more evident. PI3KC2β-KD tyrosine phosphorylation was not taken into consideration for calculating the ratio between p-Tyr and Myc-tag bands intensities. Although its phosphorylation was detected, the Myc-tag immunoprecipitation signal was not visible (Fig. 3-18 A, upper panel). All together, these results show attenuated tyrosine phosphorylation of the PI3KC2β Y127/228F mutant in comparison to the PI3KC2β-WT. According to this difference, different cellular responses were further invesigated.

In addition to phospho-status investigations, activation of the PI3K/Akt pathway was also studied upon EGF stimulation in the WCE of PI3KC2β Y127/229F HT-29 cell lines (1/15

and 5/15) in comparison to PI3KC2β-WT, -KD and -V expressing cells (Fig. 3-18 B). Increased phosphorylation of Akt on serine 473, as well as its downstream targets glycogen synthase GSK3α/β and S6 ribosomal protein was observed in all analysed cell lines upon EGF stimulation. Total Akt and S6 protein abundance seemed to be equal, whereas expression of GSK3α/β showed some discrepancies between the cell lines. In both clones of HT-29 cells over-expressing Y127/228F mutant (1/15 and 5/15) the expression level of total GSK3α/β was enhanced upon over-night starvation in 0.5% FCS and 1 h starvation in FCS-free medium, as well as in PI3KC2β-KD expressing cells (Fig. 3-18 B). PI3KC2β-WT and empty vector transfected cell lines showed equal total GSK3α/β expression independently of EGF stimulation.

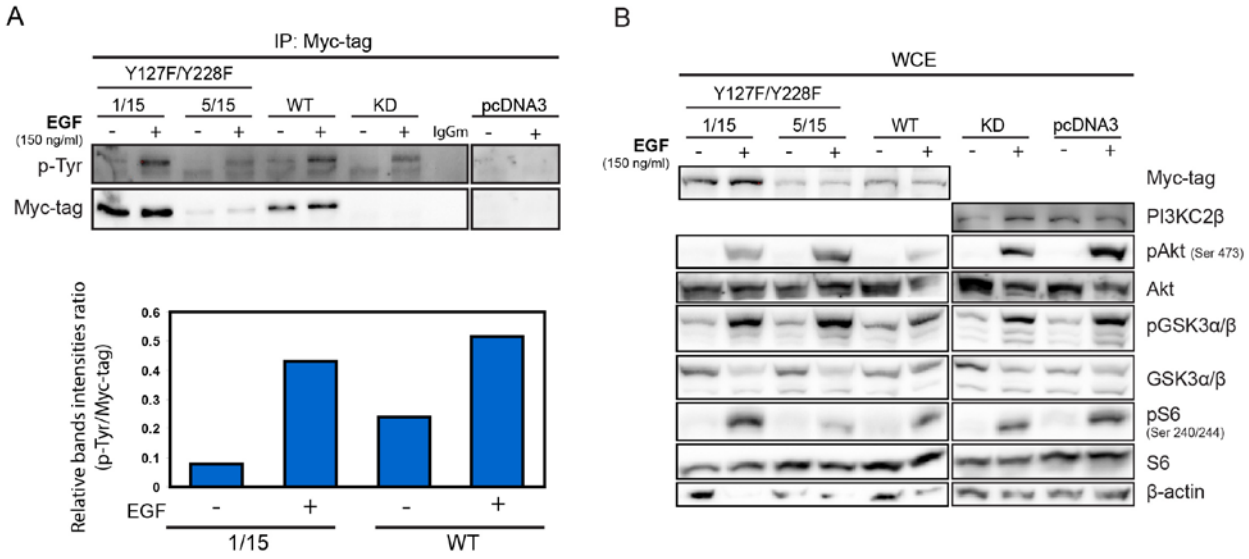

Figure 3-18. Tyrosine phosphorylation of the PI3KC2β Y127/228F mutant in the HT-29 stable cell lines. HT-29 cell lines stably expressing PI3KC2β Y127F/Y228F double mutant, wild-type (WT), kinase-dead (KD) and the empty vector (pcDNA3) were starved over-night in 0.5% FCS and then for 1 h without any FCS in the medium. Further, cells were stimulated with EGF (150 ng/ml) for 5 min. and immediately lysed on ice. Lysates were equalized for protein content and subjected to immunoprecipitation (IP) with anti-Myc-tag antibody (A). Tyrosine phosphorylation of the PI3KC2β was assessed by immunoblotting with the indicated antibodies. The level of phosphorylation was quantified by the relative p-Tyr and Myc-tag bands intensities ratio. (B) Whole cell extracts (WCE) of stimulated cells were analyzed for the exogenous PI3KC2β proteins expression and the PI3K/Akt pathway activation with the indicated antibodies. 1/15 and 5/15 represent two stable clones of the PI3KC2β Y127F/Y228F double mutant.

PI3KC2β Y127/228F mutant increases proliferation of HT-29 colon cancer cells

In order to investigate whether the differences in phosphorylation between the wild-type PI3KC2β and double mutant Y127/228F correlate with changes in cells proliferation, the MTS assay was performed in HT-29 stable cell lines in the presence of 10% FCS and upon serum starvation in 0.5% FCS containing medium (Fig. 3-19 A, B). Two stable clones of

PI3KC2β Y127/228F mutant (1/15 and 5/15) were used for this study to exclude clonal variation. The proliferation of HT-29 1/15 and 5/15 cell lines was significantly higher as compared with HT-29 PI3KC2β-WT expressing cells and this difference was observed both in medium containing high (10%) and low (0.5%) serum (Fig. 3-19 A, B). Similarly, the growth rate of HT-29 PI3KC2β-KD cells was markedly elevated, when compared to HT-29 PI3KC2β-WT cells, showing the same tendency as cells stably transfected with PI3KC2β Y127/228F double mutant. Surprisingly, there was no significant difference in proliferation between HT-29 PI3KC2β-WT and empty vector expressing cells. They were proliferating slower than the other stable cell lines and this was not dependent on the number of passages, which was similar for all generated lines. The over-expression of PI3KC2β-WT was not sufficient to cause any changes in proliferation of the HT-29 cells. Only the simultaneous loss of Y127 and Y228 phosphorylation or inactivation of PI3KC2β kinase activity induced a significant increase in cell proliferation (Fig. 3-19 A, B), indicating that PI3KC2β-mediated control of HT-29 cells proliferation is therefore equally dependent on both, tyrosine phosphorylation at residues 127 and 228 and on lipid kinase activity of the protein. Due to elevated cell proliferation response in HT-29 cells expressing PI3KC2β-KD, lack of PtdIns(3)P generation seems to be important for the obtained effects. Whether phosphorylation of tyrosine 127 and 228 directly contributes to PI3KC2β lipid kinase activity would require further investigations. Expression of both PI3KC2β Y127/228F and –KD mutants exerts a positive effect on the investigated cellular response and thus tyrosine phosphorylations of PI3KC2β N-terminal domain, as well as its catalytic activity seem to negatively regulate cell proliferation in the HT-29 cells.

The observed discrepancies between proliferation of PI3KC2β mutants (Y127/228F, KD) and PI3KC2β-WT stable cell lines were independent of time. In the 10% FCS medium HT-29 1/15 and 5/15 and HT-29 PI3KC2β-KD cells proliferated more than HT-29-C2β WT at all time points. Only the proliferation of the HT-29-C2β 1/15 cells in 10% FCS after 72 h of culture was slightly decreased (Fig. 3-19 A). For the other two time points (24 and 48 h) cell proliferation rate between the cell lines was similar in 10% serum, as well as upon starvation at 48 and 72 h (Fig. 3-19 B). At 24 h of starvation a significant difference in cell proliferation could be observed only between HT-29 PI3KC2β-KD and HT-29 PI3KC2β-WT cells.

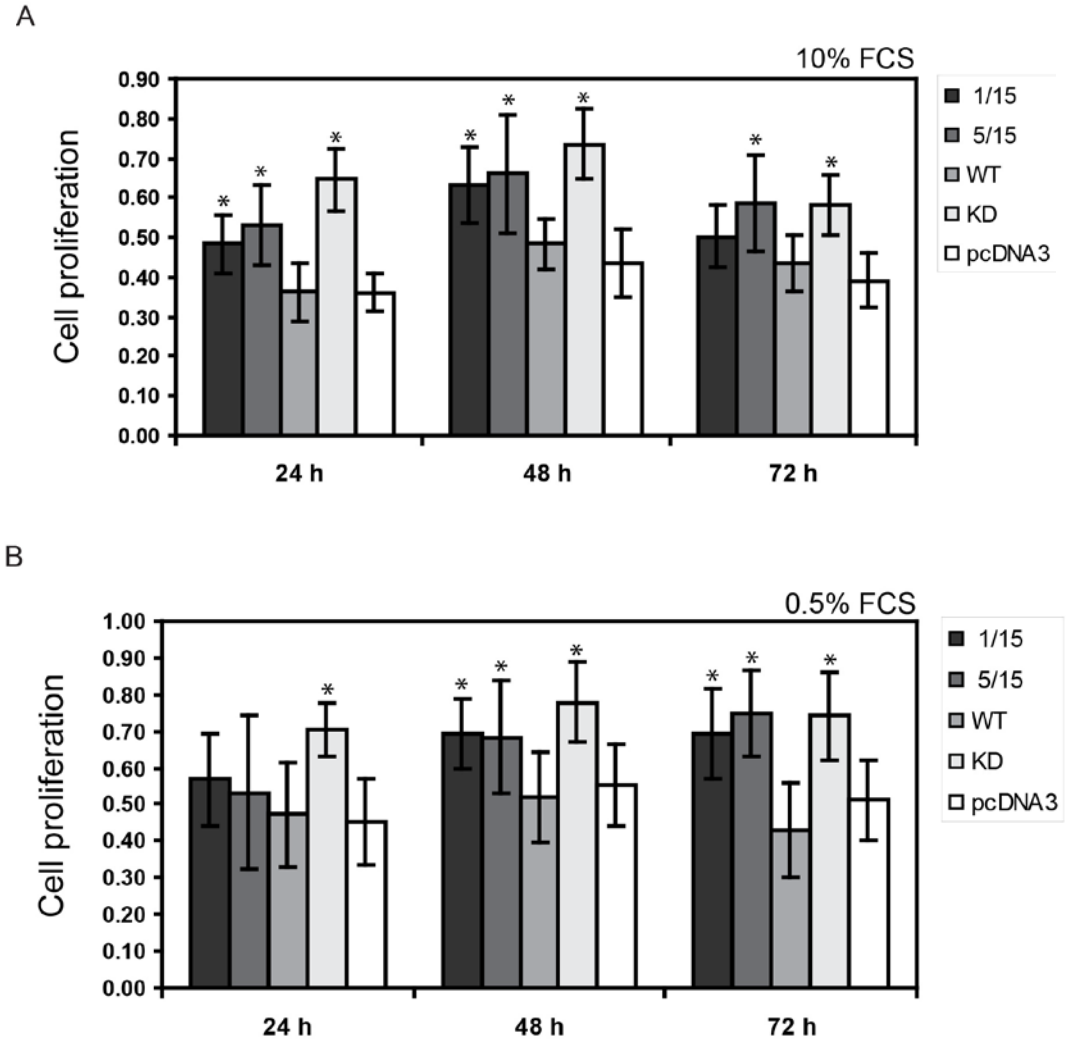

Figure 3-19. Cell proliferation of HT-29 cells stably transfected with PI3KC2β Y127/228F mutant. HT-29 cell lines stably expressing PI3KC2β Y127F/Y228F double mutant, wild-type (WT), kinase-dead (KD) and the empty vector (pcDNA3) were either cultured in 10% FCS containing medium (A) or were grown in the 10% FCS medium for 24 h and then starved in 0.5% FCS (B) for 24, 48 and 72 h, respectively. 1/15 and 5/15 represent two clones of the PI3KC2β Y127F/Y228F double mutant. Cell proliferation was assessed by the MTS cell viability assay. Data are mean ± SD from three independent experiments, *p<0.05.

PI3KC2β Y127/228F mutant induces cell-cell adhesions formation in HT-29 colon cancer cells

PI3KC2β has been shown before to be involved in the establishment of E-cadherin mediated cell-cell junctions in A-431 cancer cell of epithelial origin [103]. Therefore, in order to further understand the potential function of PI3KC2β 127 and 228 tyrosine residues in cell-cell contacts formation, the phenotypical changes in the actin cytoskeleton of the stably

transfected HT-29 cell lines were investigated. Mutation of both PI3KC2β Y127 and Y228 sites visibly increased F-actin accumulation at the cell-cell contacts, which was not observed in the case of PI3KC2β-WT transfected cells (Fig. 3-20). A similar lack of cytoskeletal changes was observed in the cells expressing the empty pcDNA3 vector. Moreover, the phenotype of PI3KC2β-KD expressing HT-29 cells was similar with the phenotype displayed by stable cell lines expressing Y127/228F PI3KC2β mutant (data not shown), suggesting the same tendency of changes, as were observed in cell proliferation assay (Fig. 3-19 A, B). This morphological difference was observed in two independent PI3KC2β Y127/228F clones (1/15 and 5/15), therefore it is unlikely that it might be due to clonal variation. These results suggest that in PI3KC2β both N-terminal pY residues (Y127, Y228), as well as its catalytical kinase activity might be important for the suppression of E-cadherin-dependent actin accumulation at the cell-cell junctions of HT-29 colon carcinoma cells. Whether the negative regulation exerted by phosphorylation of Y127 and Y228 in PI3KC2β is achieved by PtdIns(3)P synthesis or is mediated by other mechanism, which is lipid-kinase independent, is not clear at present.

In epithelial cells in culture, the localization and the cellular effect of PI3K activation depends on the degree of confluence. PI3K were shown to be recruited to newly established E-cadherin-mediated cell-cell contacts in Caco-2/15 human epithelial colorectal adenocarcinoma cells when they reached confluence [209]. Furthermore, PI3Ks seem to be more important for the assembly of cell-cell contacts than the maintenance of mature adherens junctions [209]. All HT-29 stable cell lines were grown for 72 h until they reached confluence, so the stage, when the E-cadherin-dependent cell-cell adherens junctions formation and PI3K accumulation is initiated. Interestingly, PI3KC2β Y127/228F mutant transfected cells displayed similar F-actin accumulation at the cell-cell contacts also before they reached 100% confluence, when they were still growing in compact colonies not in the monolayer of cells (according to my observations).

All these results taken together confirm previous observations that Y127 and Y228 in N terminus of PI3KC2β-WT can negatively regulate not only HT-29 cells proliferation, but also cell-cell adherens junction formation. A similar mode of regulation seems to be achieved by PI3KC2β catalytic activity, suggesting that PtdIns(3)P generation might be important. The exact mechanism of control of cell proliferation and cell-cell contacts formation would require further investigations. Whether these PI3KC2β-dependent processes are regulated in HT-29 cells by the same or different signaling pathways would also need to be studied.

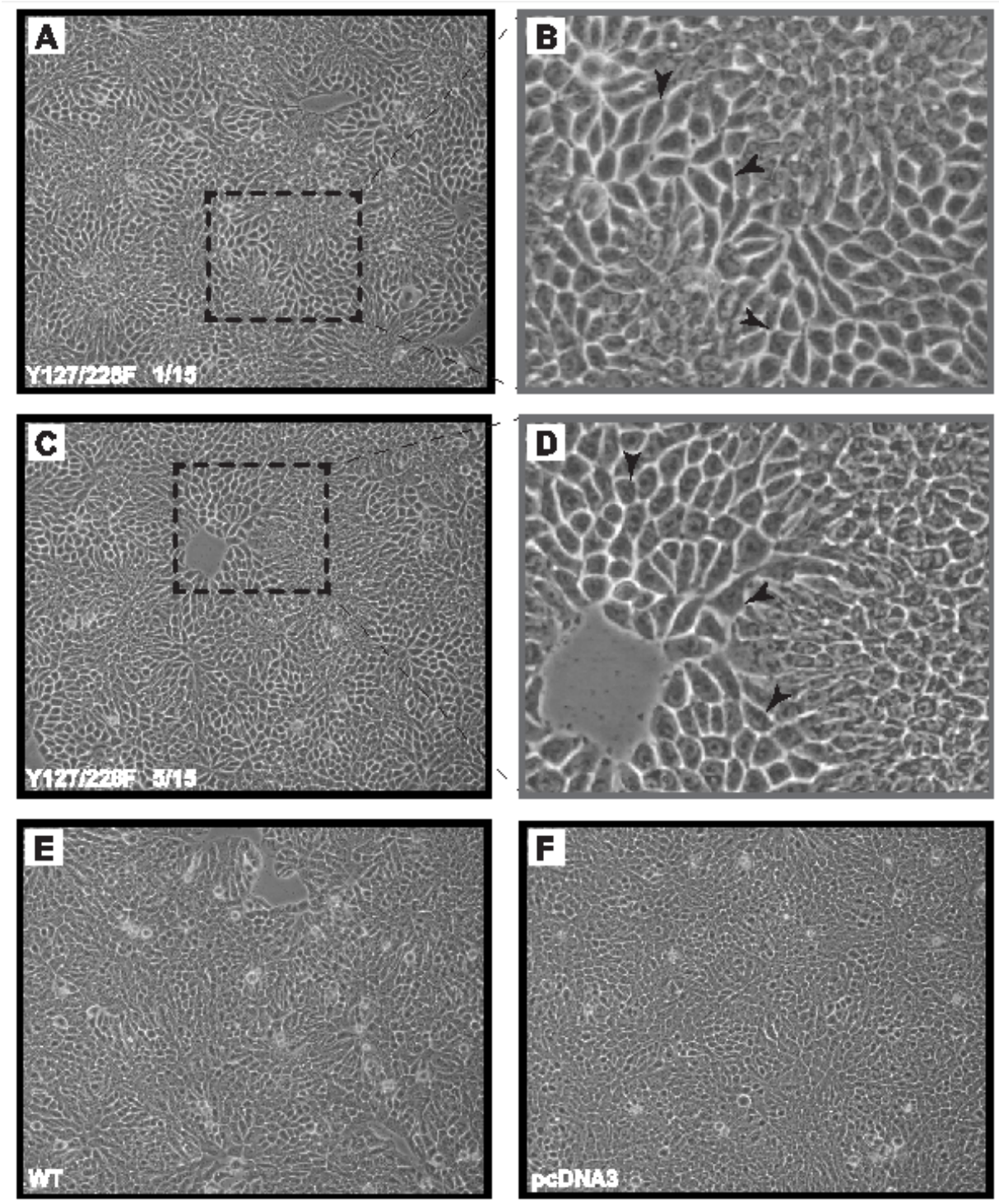

Figure 3-20. Cell-cell junction formation in HT-29 cells stably transfected with PI3KC2β Y127/228F mutant. HT-29 cell lines stably expressing PI3KC2β Y127F/Y228F double mutant, clone 1/15 (A) and clone 5/15 (C). Inset window enlarged in (B) and (D) respectively, shows F-actin accumulation at cell-cell contacts. HT-29 cell lines stably expressing PI3KC2β-WT (E) and pcDNA3 empty vector (F). All cell lines were cultured in 10% FCS containing medium for 72 h until they reached 100% confluency. Morphology pictures were taken with Eclipse TS100 inverted microscope supplied with a Nikon DXM1200 digital camera.

PI3KC2β Y127/228F mutant induces Akt/PKB signaling pathway activation in HT-29 colon cancer cells

In order to shed a light on the molecular background of the differences in cell proliferation and cell-cell contacts formation between PI3KC2β Y127/228F mutant and the wild-type expressing HT-29 cells, the activation of Akt and SAPK/JNK signaling pathways was investigated. Akt/PKB is the central regulator of cell growth, proliferation and survival, which directly binds to PI3Ks products, namely PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [25]. c-Jun N-terminal kinase/ stress actvated protein kinase (JNK/SAPK) is a downstream target of RhoGTPases, which control cytoskeletal rearrangement processes [210]. Assembly of E-cadherins requires activation of small GTPases, as well as PI3Ks, and it stimulates Akt/PKB pathway activation [146, 147, 209].

Akt pathway activation was investigated in HT-29 stably transfected cell lines upon over-night FCS starvation (0.5% FCS) and further stimulation with EGF for 10 min. Subsequently, whole cell exctracts were subjected to western blot analysis with specific antibodies. Our results revealed increased Akt phosphorylation at Ser^{473} upon EGF stimulation in HT-29 cells expressing the Y127/228F mutant, compared to PI3KC2β-WT (Fig. 3-21).

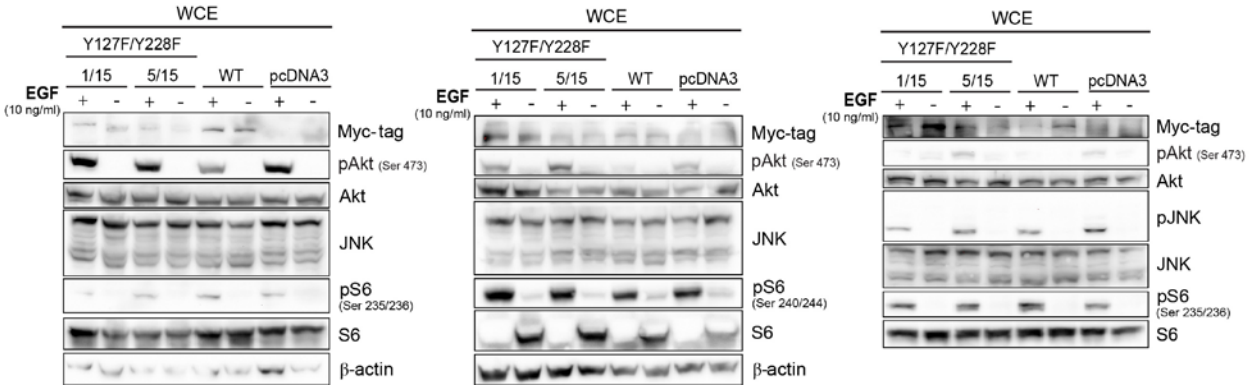

Figure 3-21. Akt/PKB signaling pathway activation in HT-29 cells stably transfected with PI3KC2β Y127/228F mutant. HT-29 cell lines (1/15 and 5/15 representing two clones) stably expressing PI3KC2β Y127F/Y228F mutant, wild-type (WT) and the empty vector (pcDNA3) were seeded and cultured in 10% FCS containing medium for 24 h. Next, they were starved over-night in 0.5% FCS and then for 1 h without FCS. Further, cells were stimulated with EGF (10 ng/ml) for 10 min. Cell lysates were equalized for protein content and subjected to immunoblotting. Activation of Akt and SAPK/JNK signaling pathways was assessed with indicated antibodies. Figure represents the results of three independent experiments.

Interestingly, Akt phosphorylation in HT-29 cells transfected with PI3KC2β-WT was decreased compared to pcDNA3 and this difference was observed in independent

experiments, also when cells were treated with higher concentrations of EGF (Fig. 3-18 B). Total Akt abundance was equal in all samples, which supports the obtained results. There were also no changes in the expression of total JNK upon EGF stimulation (Fig. 3-21), but increased phosphorylation of JNK was observed after EGF treatment. However, there were no differences in JNK phosphorylation between the investigated cell lines indicating that mutation of Y127 and Y228 in PI3KC2β do not affect JNK pathway activity. No differences between the cell lines were also observed at the level of S6 ribosomal protein phosphorylation, neither on $Ser^{236/236}$ nor on $Ser^{240/244}$, although it showed increased phosphorylation upon EGF stimulation when compared to serum-starved cells (Fig. 3-21).

3.2.4. Material and methods

Antibodies and Reagents

The following antibodies specific for indicated proteins and diluted according to the manufacturer's protocol were used: PI3KC2β was described in [72], Myc-tag (9E10) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Tyr, clone 4G10 (Upstate/ Millipore, CA, USA), phospho-Akt (Ser473), phospho-S6 protein (Ser 235/236) and (Ser 240/244), S6 protein, phospho-GSK3α/β (Ser21/9), phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK (Cell Signaling Technology, Inc., Danvers, MA, USA), Akt (Santa Cruz Biotechnology, CA, USA), GSK3α/β (Epitomics, CA, USA), β-actin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and donkey anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (1:10000 dilution) coupled with horseradish peroxidase (Amersham Biosciences).
Recombinant EGF was purchased from Calbiochem, La Jolla, CA, USA, while EcoR1, XhoI and RsrII restriction enzymes were purchased from Fermentas, Maryland, USA or EnglandBioLabs, UK.

Cell Lines

HEK293 (human embryonic kidney 293) and Ht-29 (human colon adenocarcinoma) cells were grown in the presence of 10% FCS, 1% penicillin/streptomycin and 1% of L-glutamine (v/v) in the DMEM and McCoy's medium, respectively (Life Technologies/ Invitrogen). Stably transfected Ht-29 clones were cultured in the medium supplemented with 1mg/ml of geneticin (G418). Cell cultures were maintained in a humidified atmosphere of 5% CO2 at 37°C and were passaged every 4 days.

Plasmids

The cDNA of N-terminal myc-tagged (MEQKLISEEDL) PI3KC2β wild-type was cloned into pcDNA3 vector (Invitrogen) using *EcoRI* and *XhoI* sites as described in Arcaro *et al.* 1998. Ctalytically inactive kinase-dead PI3KC2β mutant was developed by mutation of the highly conserved asparate (*D*FG) to an alanine residue in the activation domain (D1213A, DN) as described by Katso *et al.* 2006. PI3KC2β tyrosine to phenylalanine (Y/F) single and double mutants (Y127F, Y228F and Y127F/Y228F) were generated based on the above mentioned PI3KC2β wild-type construct by site-directed mutagenesis followed by mutations validation by DNA sequencing (Top Gene Technologies, Inc., Canada).

Enzymatic Restriction Digestion

Two-step digestion of PI3KC2β constructs with three different restriction enzymes was employed. 1 µg of plasmid DNA was first subjected to double digestion for 2 hrs at 37°C with EcoRI and XhoI enzymes (10U each), in appropriate restriction enzyme buffer, in total volume of 20 µl, following manufacturer's instructions (Fermentas, USA). Resulting DNA fragments were further precipitated with 3M NaAc pH=5.2 and 100% EtOH, and subjected to third digestion with RsrII (4U) enzyme (Fermentas, USA). After incubation for 2 hrs at the 37°C, digested DNA fragments were analyzed by 1.2% agarose gel electrophoresis, and stained with GelRed (Biotium, Hayward, CA, USA).

Transfections

Transient transfections of PI3KC2β phospho-tyrosine (pY) mutants, wild-type (WT) and pcDNA3 empty vector (V) were performed with Lipofectin (HEK293) and Lipofectamine LTX (HT-29) (Invitrogen) according to manufacturer's instructions.

Stable clones were derived from HT-29 cells transfected with PI3KC2β phospho-tyrosine (pY) single and double mutants (Y127F, Y228F and Y127F/Y228F) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 48 h post-transfection cells were split into selection medium containing G418 at concentration 1mg/ml. Cells were cultured in the selection medium for 2-3 following weeks. Medium was changed each 72 hours. When the single G418 resistant colonies appeared they were further selected and expanded. After 2-3 passages, expression of PI3KC2β tyrosine to phenylalanine (Y/F) mutants was verified by qPCR and western blot.

RNA Extraction and mRNA Level Analysis

Total RNA was extracetd using Rneasy Mini Kit (Qiagen, Basel, Switzerland) and transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Assays-on-Demand Gene Expression products (Applied Biosystems) were used to measure mRNA expression level of human *PI3KC2β* (Hs00898524_m1) and GAPDH (Hs99999905_m1) as a reference gene control. Relative mRNA expression levels were calculated using the comparative threshold cycle (CT) method. Semi-quantitative analysis of PI3KC2β endogenous and exogenous gene expression in HT-29 cells was performed with the One-Step RT-PCR Kit (Qiagen) with use of following primers: forward primers 5' CAGAAGCTTATTTCCGAAGAGG 3', (for exogenous cDNA expression) and 5' CTGGAAGTCCCTGGAGTCAG 3' (for endogenous cDNA expression); reverse primer 5' CTGCTTGGCTCTGTTCTCC 3' (Microsynth, Balgach

Switzerland). PCR products were stained with GelRed (Biotium, Hayward, CA, USA) and electrophoretically separated on 1.2% agarose gel.

Growth Factor Stimulation

EGF stimulation was performed to investigate tyrosine phosphorylation of endogenous PI3KC2β in HT-29 parental cells and exogenous PI3KC2β pY mutant, WT and KD in HT-29 stable cell lines. Cells were seeded and let to attach in 10% FCS containing medium for 24 hrs. Next, they were starved over-night in respectively 1% or 0.5% FCS, and then for 1 h in FCS-free medium. Further HT-29 parental cells and HT-29 stable cell lines were stimulated with EGF respectively at 50 ng/ml for 10 min. or 150 ng/ml for 5 min., washed with ice-cold phosphate-buffered saline (PBS) and immediately lysed on ice.

Cell Lysis

Cell lysates were prepared in Triton 1% buffer (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5% NP-40) supplemented with Complete Mini Protease Inhibitor Coctail (Roche Applied Sciences) and with the phosphatase inhibitors sodium fluoride (1 mM NaF), sodium ortho-vanadate (1 mM Na_3VO_4) and β-glycerophosphate (10 mM). Cell pellet was removed by centrifugation for 30 min. (15,000 rpm at 4°C). The lysates were equalized for protein content with the Thermo Scientific Pierce BCA Protein Assay Kit and subjected to immunoprecipitation or loaded on SDS-polyacrylamide gel (SDS/PAGE) as a whole cell extracts (WCE).

Western Blot

Proteins were separated by SDS/PAGE electrophoresis and immunoblotted on polyvinylidene fluoride membrane PVDF (Amersham, GE Healthcare, UK). The membranes were then blocked in 3% gelatin (for pTyr-specific antibodies) or 5% non-fat dry milk (all other antibodies) in 1×PBS over-night at 4°C. Washings were done in 1× PBS-Tween (0.1%). Incubation with the primary antibodies was performed in 4°C over-night, and with the secondary antibodies 1 h at room temperature. Chemiluminescence was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Immunoprecipitation

To investigate tyrosine phosphorylation of endogenous PI3KC2β in HT-29 parental cells and exogenous PI3KC2β pY mutant, WT and KD in HT-29 stable cell lines upon EGF stimulation immunoprecipitation with PI3KC2β and Myc-tag antibody respectively was conducted on cell

lysates equalized for protein content. Incubation with the respective primary antibodies was performed at 4 °C for 2 hrs. Protein A or G Sepharose 4 Fast Flow beads (Amersham, GE Healthcare, UK) were then added for the PI3KC2β rabbit polyclonal and Myc-tag mouse monoclonal antibodies, and the incubation was continued for 1 h at 4 °C. The immunoprecipitates were washed three times in the lysis buffer and resuspended in 2x SDS sample buffer (50 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 200mM DTT, and 0.25% bromophenol blue). Samples were denatured for 3 min. at 100°C and analysed by SDS/PAGE and western blot.

Cell Proliferation Assay

HT-29 stable cell lines were seeded in 96-well plates at a density of 5000 cells/well and grown for 24-72 hrs in high (10%) or low (0.5%) serum containing medium. For the starvation experiment cells were first seeded in 10% FCS containing medium followed by medium change with reduced FCS (0.5%) after 24 hrs. Cell proliferation was analysed by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega, Madison, WI, USA).

Microscopy

HT-29 stable cell lines wre cultured in McCoy's complete medium supplemented with G418 (1mg/ml) for 72 hrs until they reached confluency. Morphology pictures were taken under Nikon Eclipse TS100 inverted microscope (10 x magnification) supplied with a Nikon DXM1200 digital camera (Nikon ACT-1 2.70 software).

Statistical Analysis

Data are mean ± SD of three independent experiments. The GraphPad PRISM 5 software was used to calculate statistical significance of differences between groups with one-way ANOVA followed by Tukey's Multiple Comparison post tests. $P<0.05$ was considered significant and is indicated with a single asterisk.

3.2.5. Discussion

PI3KC2β-mediated control of cell proliferation

The investigation of the impact of PI3KC2β Y127 and Y228 mutations on cell growth revealed that proliferation of the HT-29-C2β 1/15 and 5/15 stable cell lines, as well as HT-29-C2β KD cells was significantly elevated in comparison to the HT-29-C2β WT cell line. On the other hand proliferation rate of the last one was comparable to HT-29-C2β V control cells in both 10% FCS medium and upon starvation, and at all time points (Fig. 3-19 A, B). These results suggest that tyrosine phosphorylation of residues 127 and 228 located in the N-terminal regulatory region of PI3KC2β may be equally important for regulation of cell proliferation signaling as the PI3KC2β-kinase activity itself. Contrasting results concerning PI3KC2β involvement in cell proliferation were published before. Whether the kinase activity increases or decreases cell proliferation seem to be cell line-specific. In HEK293 cells transfected with PI3KC2β-WT a 35% reduction of cell proliferation at day 4 was observed when compared to HEK293 cells transfected with PI3KC2β-KD and empty vector [84]. These results are consistent with my data, which show a significant increase in HT-29-C2β KD cells proliferation in comparison to HT-29-C2β WT cells. Although, in my experiments HT-29-C2β V cells behaved like HT-29-C2β WT cells and not like HT-29-C2β KD cell line. The decrease in cell proliferation of HEK293-WT cells was consistent with increased caspase-3 activity suggesting increased levels of apoptosis [84], which was not studied in context of my HT-29 stably transfected cell lines. The difference in cell growth resulting from discrepancies in cell survival was also shown in A-431 epidermoid carcinoma over-expressing PI3KC2β, which were more resistant to anoikis than A-431 parental cell line [103]. A-431-C2β WT cells demonstrated ~1.75 fold increase in cell proliferation under both low (1%) and high (10%) serum conditions, and the effect was not dependent on JNK or Akt signaling [103]. On the other hand, inhibition of endogenous PI3KC2β with the isoform-specific pharmacological inhibitors (PI701, PI702) in AML and SCLC cell lines, which highly express the kinase, resulted in impaired cell proliferation [129]. These results were further confirmed with PI3KC2β siRNA down-regulation in AML cell lines, where reduced cell proliferation was further accompanied by increased caspase-3 activity [129]. According to the above studies, over-expression of PI3KC2β-WT or -KD either increases or decreases cell proliferation. It is possible that differential regulation exists in various cell lines. In this context, a decrease in cell proliferation in HT-29-C2β WT cells when compared to HT-29 stable cell lines expressing Y127/228F or kinase-dead PI3KC2β mutant is therefore possible. However, explaining why HT-29 cells transfected with PI3KC2β-WT and empty vector (V) as control behave similar is

a difficult issue, since in the cited studies the difference in HEK293 cell proliferation between –WT and –V expressing cells was detected [84]. It should not be forgotten that the newly generated HT-29 stable cell lines still express a minimal level of endogenous PI3KC2β. Exogenous expression of PI3KC2β-WT increased the protein level in the cells, but this higher expression was not sufficent to induce any change in cell proliferation when compared to PI3KC2β-V (Fig. 3-19 A, B). The reasons for that might be multiple. For instance, although PI3KC2β-WT level is elevated in the cell, co-expression of the PI3KC2β-associated proteins would be necessary to exert an effect on cell proliferation. This and other issues would certainly require further experimental verification.

PI3KC2β-mediated control of cell-cell adhesion

Involvement of PI3Ks in E-cadherin-mediated cellular aggregation and thus Akt/PKB signaling activation was documented previously in epithelial cells [147, 209]. In response to cell-cell contact formation a physical interaction between PI3K and E-cadherin-containing complexes was observed in immunoprecipitation experiments performed in MDCK cells [147]. PI3KC2β engagement in F-actin and E-cadherin cell-cell junction formation was furthermore reported [103]. Over-expression of PI3KC2β-WT in the human epithelial carcinoma cell line A-431 induced increase in F-actin and E-cadherin at the sites of cell-cell contacts, while the PI3KC2β-KD mutant expressing cells lost the ability to stabilize cell-cell adhesion. The cells were contacting with each other via filopodia and they were much less compact than cells transfected with PI3KC2β-WT [103]. These results are in contrast to what I observed in HT-29 colon carcinoma cell lines stably transfected with PI3KC2β-WT or -KD mutant. PI3KC2β-WT expressing cells were compact, but generation of F-actin cell-cell adhesions was not observed as it was seen in cells transfected with kinase-dead form of the protein (Fig. 3-20, KD not shown). Both of the cancer cell lines, A-431 and HT-29 are carcinoma cells, which originate from epithelial tissues. According to that, transfection of PI3KC2β forms induces changes in cell-cell adhesion, although the effects of PI3KC2β-WT and –KD expression in these cells are different. Again, these discrepancies may result from the different genetic backgrounds of A-431 and HT-29 cells and might be cell-line specific effect, as suggested in the previous section. HT-29 cell line is an EGFR-positive, *KRAS* wild-type, *BRAF*-mutated, as well as *APC* tumor supressor gene-mutated cell line, whereas A-431 cells harbor wild-type EGFR gene amplification and *KRAS* and *BRAF* wild-type versions [211, 212]. TP53 tumor supressor gene is not functional in both cell lines leading to increased sensitivity to mitogenic stimuli. However, due to EGFR over-expression, the response of A-431 cells to ligand stimulation might be much more rapid, although nanomolar

concentrations of EGF were shown to inhibit A-431 cell growth, whereas picomolar concentrations promoted it [213]. On the other hand, in the HT-29 cell line missense mutations of p110α were found (COSMIC data base, http://www.sanger.ac.uk). Mutations in *PI3KCA* are known to be one of the most frequently found genetic alterations in different human cancers including colon tumors [214]. Often they lead to over-activation of PI3K/Akt pathway and related pathways, which leads to tumor progression.

A possible mechanism of PI3K activation in cell-cell junctions formation was proposed [147]. It was observed that tyrosine phosphorylation pattern of the p85 regulatory subunit of class I PI3K corresponded to the p85 complex formation with E-cadherin in MDCK (Madin-Darby canine kidney cells) upon cellular aggregation [147]. It was therefore suggested that a specific tyrosine kinase may exist, which phosphorylates p85/PI3K and is activated in response to E-cadherin mediated cell-cell adhesions formation. Furthermore, this kinase might facilitate the recruitment of PI3-kinase to E-cadherin-containing complexes at the level of the plasma membrane [147]. PI3KC2β does not associate with a regulatory subunit, but instead contains N-terminal extension, which was shown to play a regulatory role for the protein catalytic activity [93]. In the mass spectrometry analysis in my studies the PI3KC2β N-terminus was found to be tyrosine phosphorylated. It is therefore possible that PI3KC2β might be also activated upon formation of cell-cell contacts in epithelial cells. Over-expression of the wild-type form of the kinase in HT-29 cells did not cause any significant changes in the actin cytoskeleton, whereas mutation of Y127 and Y228 induced F-actin accumulation at the cell adhesions, suggesting that phosphorylation of these residues is important for the fine-tuned regulation of the cell-cell contacts formation (Fig. 3-20). How the PI3KC2β-mediated negative regulation mode in HT-29 cells is controlled is not know. Tyrosine phosphorylation of p85/PI3K stimulates PI3K activity [215]. Phosphorylation of Y127 and Y228 in PI3KC2β in HT-29 cells seem to play an opposite role based on the observed cellular phenotype. However, which kind of mechanism is involved in this process is unknown. Taking into consideration similar phenotypical changes acquired upon PI3KC2β-KD expression in HT-29 cells, the involvement of the enzyme's kinase activity should be taken into account.

p85 phosphorylation is catalyzed by Src-family protein-tyrosine kinases Abl and Lck at the Y688 [216], while the kinase, which may phosphorylate Y127 and Y228 in PI3KC2β was not studies. However, previous observations from our lab suggested that it could be the Src non-receptor tyrosine kinase, which actually is expressed at the high levels in the HT-29 cells [217]. This hypothesis however would need to be confirmed.

Influence of PI3KC2β Y127/228F mutant on Akt/PKB pathway activation

My signaling data cannot be fully compared to the cell proliferation and cell morphology studies since cells in these assays were not treated with EGF. However, it is important to mention that E-cadherin engagement in cell-cell adhesions stimulates Akt and p38 pathways through PI3K-dependent mechanism [209]. Mutation of Y127 and 228F in PI3KC2β sequence induced increased activation of the Akt/PKB signaling pathway in HT-29 cells upon EGF stimulation when compared to PI3KC2β-WT (Fig. 3-21). On the other hand, Akt phosphorylation levels in HT-29 cells transfected with PI3KC2β Y127/228F mutant were not different from the levels of Akt phosphorylation in empty vector-transfected cells. Again, this confirms that the newly identified PI3KC2β tyrosine phosphorylation sites negatively regulate the enzyme activation and downstream signaling, but also suggests that the enzymatic function of PI3KC2β Y127/228F mutant was affected due to mutation. A similar effect was already observed in HEK293 cells transfected with pcDNA3, PI3KC2β-WT and -KD enzyme [84]. Cells were starved over-night and then stimulated with 10% FBS containing medium for 4-24 hrs. Starting from 16 hrs phosphorylation of Akt on Ser^{473} and Thr^{308} was significantly blunted in HEK293 cells over-expressing PI3KC2β-WT, while in the empty vector and PI3KC2β–KD expressing cells it was sustained at the same level. According to authors, an analogous effect was obtained when the same HEK293 cells were stimulated with EGF, but with much shorter kinetics. Under these conditions, Akt phosphorylation was attenuated more rapidly in cells over-expressing PI3KC2β-WT and it correlated with lower intracellular levels of PtdIns(3,4,5)P_3 [84]. Analogously, HT-29 cells expressing PI3KC2β Y127/228F mutant or empty vector showed increased Akt phosphorylation in comparison to cells transfected with PI3KC2β-WT (Fig. 3-18 B and 3-21). Unfortunately, the intracellular levels of phosphoinositides were not investigated. The authors of the above studies point out the strong time contrast between FBS and EGF stimulation. They also emphasized decreased levels of PtdIns(3,4,5)P_3 and increased levels of PTEN in HEK293 cells over-expressing PI3KC2β-WT suggesting that PI3KC2β might regulate class I PI3K signaling [84]. This interesting hypothesis is partially confirmed in my results. However, Akt/PKB directly binds to both PI3Ks products, namely PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [25]. The main product of PI3KC2β activity *in vitro* is PtdIns(3)P, but under some circumstances (in the presence of Mg^{2+}) it can potentially generate PtdIns(3,4)P_2 [72]. Whether PI3KC2β-mediated regulation of Akt activity is due to class I PI3Ks activation or other factors, is an interesting question. Moreover, no difference in Akt phosphorylation at Ser^{473} was detected between A-431 cells over-expressing PI3KC2β-WT and parental A-431 cells. No Akt impact on cell proliferation or protection against anoikis was observed [103]. More studies would be therefore required to

find out how PI3KC2β regulates Akt signaling in HT-29 cells and whether it is through a direct or indirect mechanism.

Mechanism of regulation PI3KC2β function by Y127 and Y228 phosphorylation in the N-terminal domain

Here, I identified that phosphorylation of Y127 and Y228 in PI3KC2β negatively regulates HT-29 cell proliferation and cell-cell adhesions formation. This mode of action was reflected in the Akt/PKB signaling pathway activation upon stimulation with EGF, where Akt phosphorylation at Ser^{473} was found to be down-regulated in PI3KC2β-WT expressing cells when compared to HT-29 cells over-expressing PI3KC2β Y127/228F mutant, as well as an empty vector control. These results are in line with recently published reports, which identifiy PI3KC2β's role in negative regulation of Akt phosphorylation in HeLa cells and primary human skeletal muscle myotubes [218]. PI3KC2β generates a pool of PtdIns(3)P, which becomes a substrate for myotubularin 1 (MTM1) phosphatase. This in turn has the ability to dephosphorylate PtdIns(3)P and to generate PtdIns, which seems to stimulate Akt/PKB pathway. Down-regulation of MTM1 results in the accumulation of PtdIns(3)P and inhibition of the EGF-induced Akt phosphorylation, as well as its downstream targets (4E-BP1, FoxO transcription factors, 70S6K), which leads to caspase-dependent cell death. These findings unveil PtdIns(3)P's role as a direct negative regulator of Akt/PKB signaling pathway. Co-silencing of PI3KC2β in MTM1 down-regulated HeLa cells reduced PtdIns(3)P level and restored EGF-mediated Akt phosphorylation clearly indicating an importance of PI3KC2β lipid product in negative regulation of Akt activation. MTM1 on the other hand acts as a positive reulator of Akt-dependent cell growth and survival by converting PtdIns(3)P to PtdIns [218]. A schematic representation of PI3KC2β and MTM1-mediated regulation of Akt/PKB pathway activation by modulation of PtdIns(3)P levels upon EGF stimlation can be found in Fig. 3-22 A-C. These data are consistent with my results, which show decreased Akt phosphorylation upon HT-29 cell transfection with PI3KC2β-WT when compared to PI3KC2β Y127/228F mutant and empty vector transfected cells (Fig. 3-21). It could therefore suggest that the PtdIns(3)P pool generated by PI3KC2β may negatively regulate Akt/PKB pathway activation in HT-29 cells. In cells transfected with PI3KC2β-WT there is still some level of endogenous protein, which additionally contributes to the observed effect. In HT-29 cells expressing an empty vector or un-functional PI3KC2β Y127/228F mutant, synthesis of additional pools of negative regulator PtdIns(3)P is missing, therefore Akt activation increases. This hypothesis could be also true for PI3KC2β-KD transfected cells.

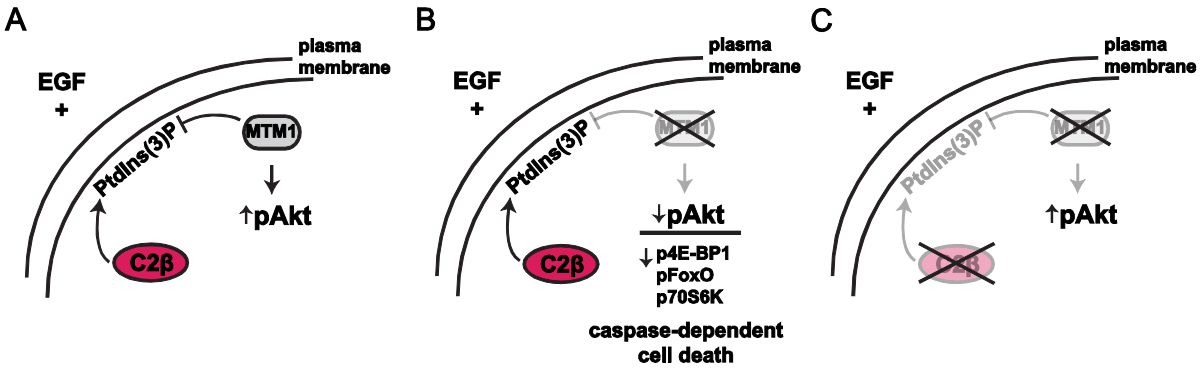

Figure 3-22. PI3KC2β and MTM1 – mediated regulation of Akt/PKB pathway activation. (A) Upon EGF stimulation PI3KC2β generates PtdIns(3)P pool in HeLa cells, which becomes a substrate for MTM1 (myotubularin 1) phosphatase. As a result of PtdIns(3)P dephosphorylation, PtdIns is generated, which seem to stimulate Akt/PKB pathway activation. (B) Knock-down of MTM1 increases PI3KC2β-dependent PtdIns(3)P level, which neagtively regulates phosphorylation of Akt and its downstream targets leading to caspase-dependent cell death. (C) Co-silencing of PI3KC2β in MTM1 down-regulated HeLa cells decreases PtdIns(3)P pool and restores EGF-mediated Akt activation.

The tyrosine phosphorylation sites 127 and 228 may therefore directly control PI3KC2β activity, which leads to increased PtdIns(3)P synthesis. In other words, Y127 and Y228 phosphorylation positively regulates PI3KC2β catalytic activity toward PtdIns leading to generation of PtdIns(3)P, which negatively regulate activation of Akt/PKB and associated signaling pathways (schematic representation of the hypothetic model, see Fig. 3-23).

Most likely, N-terminal phosphorylation of Y127 and Y228 is necessary for appropriate regulation of PI3KC2β lipid kinase activity. Moreover, it has been proven that regulatory role of the N terminus in PI3KC2α and PI3KC2β strongly depends on the binding of additional molecular factors such as clathrin or intersectin [92, 93, 104]. Therefore, in HT-29 cells a non-receptor tyrosine kinase may exist, which interacts with the enzyme leading to its phosphorylation and activation. On the other hand, the possibility exists that phosphorylation of Y127 and Y228 indirectly controls PI3KC2β function through cross-talk with other signaling molecules and has nothing to do with enzyme's kinase activity toward lipids in the membrane. Not only proline-rich motifs in the N-terminal portion of the kinase can play a role of docking sites, but also phospho-tyrosine residues themselves can act as binding motifs for multiple SH2 or PTB-containing proteins [18]. As already shown for other PI3Ks [219], PI3KC2β might be involved in protein-protein interactions that affect signaling independently of its kinase activity. Thus, it may play a role of scaffolding protein transmiting signals to other effector molecules and simultaneously it stays inactive as a kinase. However, since I observed similar cellular responses for HT-29 cells transfected with PI3KC2β-KD and with the PI3KC2β Y127/228F mutant (Fig. 3-19 A, B), it is more likely that phosphorylation of 127

and 228 tyrosine residues controls lipid kinase function of PI3KC2β and that PtdIns(3)P pools may play an important role in cell proliferation and cell-cell adhesions formation.

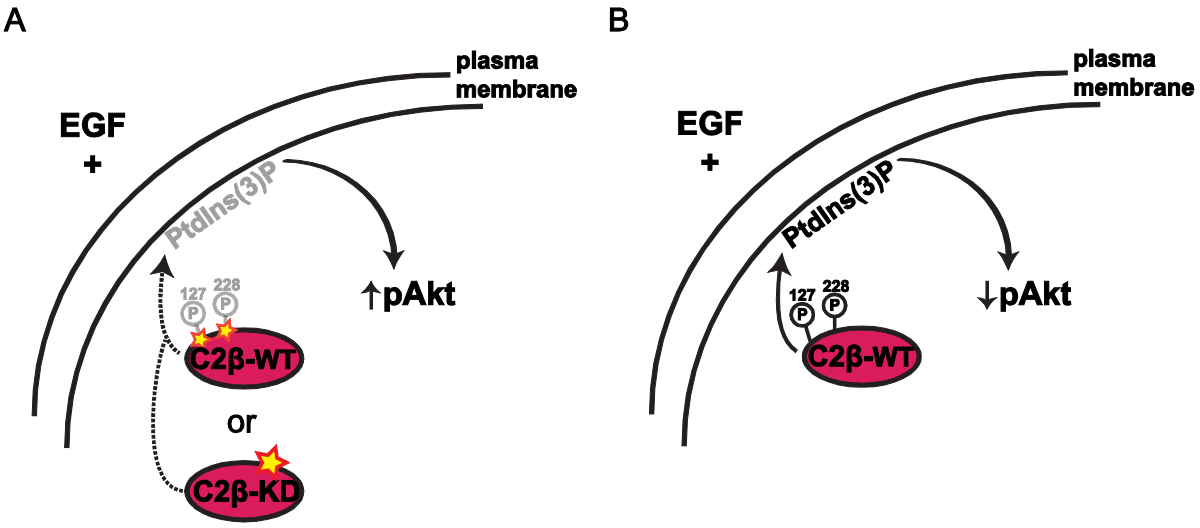

Figure 3-23. Hypothetical model illustrating PI3KC2β negative regulation of Akt/PKB pathway in HT-29 cells. (A) Mutation of Y127 and Y228 in PI3KC2β-WT or inactivation of PI3KC2β catalytic domain leads to decrease in PtdIns(3)P levels at the plasma mebrane. Loss of PtdIns(3)P, Akt negative regulator, increases Akt/PKB activation. (B) Phosphorylation of Y127 and Y228 in N-terminal region of PI3KC2β regulates the enzyme's kinase activity toward PtdIns leading to generation of PtdIns(3)P, which in turn suppress Akt phoshorylation. WT–wild-type, KD–kinase-dead. Stars indicate mutation.

Production of specific phosphorylated lipids in the membranes of different cellular compartments is a dynamic process resulting from orchestrated action of lipid kinases and phosphatases. In comparison to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, substantial quantities of PtdIns(3)P are present in the cells in the basal state and does not increase much upon stimulation [25], suggesting a general role of PtdIns(3)P in maintaining cellular homeostasis. Class II enzymes supported by the activation of lipid phosphatases may therefore be responsible for keeping cellular processes in balance. With respect to that, a possible role of MTM1 in PI3KC2β signaling in HT-29 stable cell lines could be verified too. It was shown in HeLa cells that knock-down of PI3KC2β, but not PI3KC2α or Vps34 counteracted the effect of MTM1-depletion on Akt phosphorylation and apoptotic pathway [218]. Moreover, it was previously demonstrated in *Drosophila melanogaster* that Pi3K68D, but not Vps34, acts in collaboration with endosomal Mtm-1 to coregulate the PI(3)P pool, which in turn promotes F-actin reorganization and modulates protrusion formation in hemocytes and insect immune cells. Human *Mtm* genes present high functional conservation with the *D. melanogaster* MTM

family members, therefore it is not surprising that MTM/Class II PI3K pathway may be important for similar biological responses in mammalian cells [220].

The increase in cell proliferation and cell-cell adhesions formation in PI3KC2β Y127/228F and –KD mutants upon 72 hrs of culture in 10% serum is also an intriguing issue. E-cadherin was in fact associated with both anti-proliferative role, as well as increased cell proliferation depending on the cellular system, adhesive context and seeding density [221]. In colon carcinomas proliferation was linked with the localization of E-cadherin to the cell periphery [222]. The assembly of adherens junctions is promoted by PI3Ks, which in turn control Akt and p38 MAPK activation [209], but it also involves Rho GTPases [221]. Therefore, it would be interesting to investigate through which signaling pathways cell proliferation and cell-cell adhesions formation are regulated in HT-29 stably transfected with PI3KC2β Y127/228F and –KD mutants.

PI3KC2B somatic mutations in cancer

Amplifications and over-expression of *PIK3C2B* in different types of human cancer were identified, but the significance and consequences of these alterations have not been studied in detail. Even less information is available about *PIK3C2B* mutations in tumors, but some reports have started to appear due to increasing cancer cells genome sequencing data. Frequent *PIK3C2B* mutations were found recently in NSCLC adenocarcinomas and squamous cell carcinomas [127]. They were not found in previous sequencing studies, which determined already *TP53, KRAS, EGFR, CDKN2A* and *RB1* to be the most common mutated genes in lung cancer. *PIK3C2B* alterations were detected in the exons and appeared to be missense mutations predicted to affect protein function [127]. However, how these mutations contrubute to the lung cancer progression has not been studied yet. The situation is different with the identification of SNPs in the *PIK3C2β* gene, whose function was significantly associated with prostate cancer risk, especially for men diagnosed before age of 65 or for men with a family history of prostate cancer [142]. On the other hand, my results showed that mutation of the tyrosine phosphorylation sites in N-terminal region of PI3KC2β can lead to deregulation of cell-cell adhesion processes in epithelial colon cancer cells (Fig. 3-20). Whether these types of PI3KC2β mutations are present in tumors during the process of mutation accumulation is not known. However, the increasing amount of cancer genomes sequencing data may still bring an answer to that question. While discussing the increased cell-cell adhesions formation and elevated cell proliferation induced by mutation of Y127 and Y228 in PI3KC2β-WT, it is good to remember that perturbation of intracellular adhesion is directly implicated in carcinogenesis and the onset of most solid tumors [148]. First, cancer

cells have to break cell-cell and cell-matrix adhesions to be able to proliferate or migrate to distinct tissues. Furthermore, they need to preserve their phenotypic plasticity to transit through the bloodstream or lymphatic vessels, and renew proliferation in secondary sites of the body [148]. The breaking and reassembling PI3KC2β-regulated E-cadherin cell-cell connections or cell-matrix adhesions might be therefore essential at each phase of metastasis. It is noteworthy that PI3KC2β expression was recently correlated with metastasis in oesophageal squamous cell carcinoma (ESCC) patients [141]. 68.2% of patients with PI3KC2β-positive ESCC tumors had metastasis compared with only 31.8% of PI3KC2β-negative cases [141].

3.2.6. Conclusions and outlook

The aim of this project was to characterize the functional relevance of tyrosine phosphorylation sites identified within PI3KC2β isolated from human cancer cell lines, which were treated with or without pervanadate/calyculin, 10% serum or EGF. Mass spectrometry analysis of cell lysates revealed four new tyrosine phosphorylation sites, namely Y68, Y127, Y228 and Y1541, which were located at the regulatory N-terminal and C2 C-terminal domains of the kinase. All of them appeared to be conserved throughout evolution among different vertebrate species suggesting their functional importance. Indeed, loss of Y127 and Y228 phosphorylation due to site-directed mutagenesis within the N terminus of the PI3KC2β-WT construct induced increased proliferation and cell-cell junctions formation when compared to the wild-type form of PI3KC2β in HT-29 stably transfected colorectal carcinoma cells. Similar results were obtained for HT-29 cells expressing PI3KC2β-KD, which indicated an important role of the PI3KC2β catalytical activity in the investigated cellular responses. Additionally, a decrease in Akt phosphorylation at Ser^{473} was observed in HT-29 cells over-expressing PI3KC2β Y127/228F or –KD mutant when comapred to PI3KC2β-WT expressing cells. Therefore, I propose that phosphorylation of Y127 and Y228 negatively regulates Akt/PKB pathway activation, and in turn cell proliferation and cell-cell junctions formation. The mechanism of this regulation remains unknown. However, I hypothesize that PtdIns(3)P, as PI3KC2β's main lipid product, may play a function of negative regulator, as showed previously in HeLa cells [218]. In this context, phosphorylation of Y127 and Y228 in PI3KC2β may positively regulate kinase activity of the enzyme leading to generation of PtdIns(3)P, which in turn negatively regulates Akt and associated downstream signaling pathways, which further helps to maintain the balance in functioning of the cell. Whether the N-terminal tyrosine phosphorylation operates in concert with the kinase activity or act in complete autonomy from the catalytic function would be an interesting issue for further investigations.

To confirm the proposed hypothesis, the first and the most important thing to test would be to investigate PtdIns(3)P synthesis in HT-29 cells stably transfected with PI3KC2β Y127/228F or -KD mutants, -WT and empty vector. This would give an answer to the question of whether phosphorylation of Y127 and Y228 is essential for stimulation of PI3KC2β enzymatic function. Furthermore, PtdIns(3)P is not a direct ligand of the Akt PH-domain, suggesting the involvement of other mediators in the Akt activation. Whether these are class I PI3Ks or other factors would be interesting to find out. Subsequently, more detailed studies of signaling pathways activated upon PI3KC2β mutations would be required. The possible involvement of Rho GTPases or MAPK pathways would have to be examined, as well as investigating whether cell proliferation and cell-cell adhesions formation are controlled by the same or different signaling pathways. Studying the possible physical

interaction of PI3KC2β with E-cadherin would help understand the mechanism of cell-cell contacts formation in PI3KC2β Y127/228F and –KD transfected cells. Last but not least, dissecting Y127 and Y228's role in the described cellular responses would bring even more understanding into the process of PI3KC2β regulation in human cancer cells.

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10/2001 – 06/2006 **Poznań University of Life Science, Poland**
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MSc thesis: *"Composition of culture medium for in vitro maturation and incidence of apoptosis in bovine blastocysts"*
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Revealing the mechanism and the role of phosphoinositide 3-kinase C2β in cytoskeletal organisation and cell migration. Blajecka K, Marinov M, Arcaro A.

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Swiss Society of Biochemistry

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7. APPENDIX

Review article published together with my colleauge during the course of my studies

Phosphatidylinositol 3-Kinase Isoforms as Novel Drug Targets

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Phosphatidylinositol 3-Kinase Isoforms as Novel Drug Targets

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Abstract: Phosphatidylinositol 3-kinases (PI3Ks) are key molecules in the signal transduction pathways initiated by the binding of extracellular signals to their cell surface receptors. The PI3K family of enzymes comprises eight catalytic isoforms subdivided into three classes and control a variety of cellular processes including proliferation, growth, apoptosis, migration and metabolism. Deregulation of the PI3K pathway has been extensively investigated in connection to cancer, but is also involved in other commonly occurring diseases such as chronic inflammation, autoimmunity, allergy, atherosclerosis, cardiovascular and metabolic diseases. The fact that the PI3K pathway is deregulated in a large number of human diseases, and its importance for different cellular responses, makes it an attractive drug target. Pharmacological PI3K inhibitors have played a very important role in studying cellular responses involving these enzymes. Currently, a wide range of selective PI3K inhibitors have been tested in preclinical studies and some have entered clinical trials in oncology. However, due to the complexity of PI3K signaling pathways, developing an effective anti-cancer therapy may be difficult. The biggest challenge in curing cancer patients with various signaling pathway abnormalities is to target multiple components of different signal transduction pathways with mechanism-based combinatorial treatments. In this article we will give an overview of the complex role of PI3K isoforms in human diseases and discuss their potential as drug targets. In addition, we will describe the drugs currently used in clinical trials, as well as promising emerging candidates.

Keywords: Phosphatidylinositol 3-kinases (PI3Ks), cancer, autoimmune and cardiovascular diseases, ATP-competitive small molecule inhibitors.

1. INTRODUCTION

The involvement of phosphatidylinositol 3-kinases (PI3Ks) in human diseases has been known for over two decades, and the importance and knowledge concerning the PI3K/Akt signaling pathway complexity appears to increase each year. The PI3K/Akt pathway is deregulated in many human diseases and in the majority of human cancers, and is therefore an attractive target for therapeutic intervention. In this review we will highlight the importance of PI3Ks for normal cell signaling and its involvement in oncogenesis and other diseases, and finally discuss its potential as therapeutic target. Furthermore, we will review the data concerning the new and available pharmacological inhibitors targeting this key signaling pathway.

1.1. The PI3K/Akt Pathway – An Overview

PI3Ks are a family of evolutionary conserved lipid kinases, playing a crucial role in controlling a wide variety of intracellular signaling events. Therefore PI3K signaling is often found deregulated in human diseases, not at least in cancer. Activation of the PI3K/Akt pathway leads to increased cell growth, increased proliferation, protection against apoptosis, cell migration, and controls vesicular transport and actin rearrangements [1].

The PI3Ks are activated in response to extracellular stimuli and receptor stimulation. Upon activation, the PI3Ks phosphorylate plasma membrane lipid phosphatidylinositoles at the 3' OH-group of the inositol ring, thereby producing distinct second messengers such as PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (PIP_3). These lipid products are known to activate and recruit a diversity of target proteins involved in complex signaling cascades. The effector proteins are recruited to the plasma membrane by binding to the PtdIns via the pleckstrin homology domain (PH-domain), phox domain (PX) or FYVE zinc finger domains of the proteins [2, 3].

One of the most important proteins activated by PIP_3 is the enzyme phosphoinositide-dependent protein kinase-1 (PDK1), which controls the activation of the key signal transducer protein kinase B (PKB)/Akt. These two PH domains-containing serine/threonine kinases, (PDK1 and Akt) are brought into close proximity at the cell membrane by PIP_3, where PDK1 activates Akt by phosphorylation at threonine 308 [4, 5]. However, to become fully activated Akt additionally needs to become phosphorylated at serine 473 by the mammalian target of rapamycin complex 2 (mTORC2) [6]. Upon activation, Akt phosphorylates a wide range of target proteins which control apoptosis, cell survival, growth and proliferation (Fig. **1**). Akt influences the cell cycle and glucose metabolism through glycogen synthase kinase-3 beta (GSK3β) [7] and modulates cell growth and survival, as well as controls the translational machinery through the mammalian target of rapamycin complex 1 (mTORC1) [8], the ribosomal protein S6 kinase (S6K) and the 4E-binding protein (4E-BP) [9, 10]. Additionally, Akt regulates cell survival by phosphorylating the forkhead human rhabdomyosarcoma transcription factor (FKHR), and thereby inhibiting the translation of pro-apoptopic genes such as the Bcl-2-antagonist of cell death (BAD), Bcl-2-interacting mediator of cell death (BIM) and

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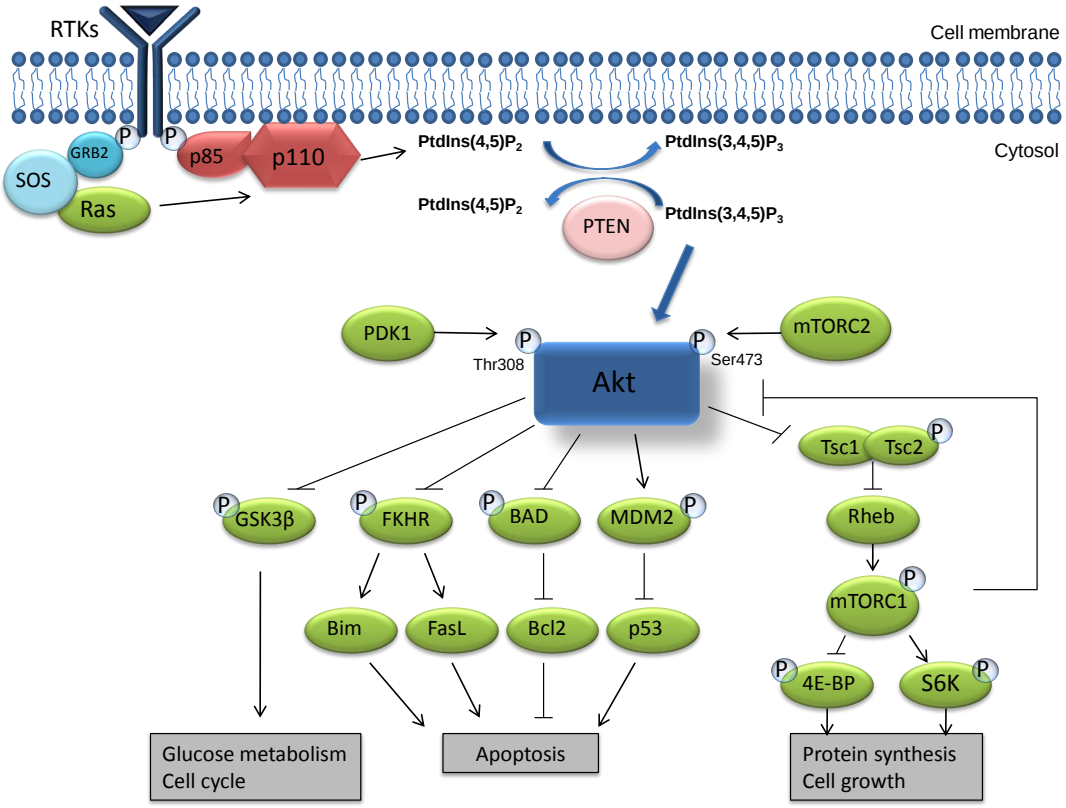


Fig. (1). The PI3K/Akt signaling cascade. Upon ligand stimulation, RTKs becomes autophosphorylated and recruits the p85-p110 heterodimer to the plasma membrane. In consequence, the PI3K complex phosphorylates PtdIns(4,5)P_2 located in the membrane, producing PtdIns(3,4,5)P_3 which acts as a docking site for PH-domain containing proteins, such as Akt and PDK1. Subsequenty, PDK1 together with mTORC2 phosphorylates Akt at Thr308 and Ser473, respectively. Activation of Akt induces a cascade of signaling events controlling glucose metabolism, cell cycle, apoptosis, protein synthesis and cell growth. Moreover, a negative feedback loop from mTORC1 and S6K to PI3K and Akt blocks pro-survival and proliferative signaling. The tumor suppressor PTEN antagonizes the action of PI3Ks by dephosphorylating PtdIns(3,4,5)P_3.

Fas ligand (FasL) [11, 12]. Akt furthermore phosphorylates the murine double minute 2 protein (Mdm2), which antagonizes p53-mediated apoptosis. The PI3Ks can additionally be activated independently of upstream regulators when the tumor suppressor protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is inactivated through mutation. PTEN is an antagonist of PI3Ks which can dephosphorylate the 3' position of PIP_3 and therefore acts as a brake for class I PI3Ks. *PTEN* is therefore not surprisingly often found mutated in cancer, leading to constitutive activation of the PI3K/Akt signaling axis [13-25].

2. PI3K CLASSIFICATION

The family of PI3Ks consists of 8 catalytic isoforms in human and is subdivided into three classes (class I, II and III) according to their sequence homologies and *in vitro* substrate preference (Box **1**) [26]. Class I is further subdivided into class I_A and class I_B depending on the receptors they become activated by. Of the three classes, class I PI3Ks are the best characterized in human diseases, while the role of class II and III is less well defined. We will therefore focus our discussion mostly on the class I PI3Ks.

2.1. Class I_A PI3K Isoforms

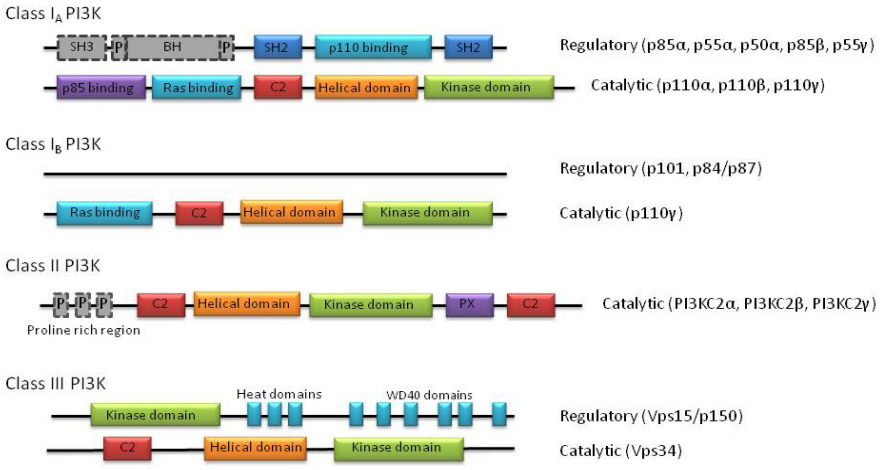
Class I_A PI3Ks comprises a group of catalytic (p110α, p110β, p110δ) and regulatory (p85α, p85β, p55δ, p55α, p50α) subunits which become activated through direct interaction with a broad variety of receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor-1 receptor (IGF-1R/IRS) [27-31]. On the other hand, the p110β isoform of class I_A together with p110γ from class I_B is regulated by G-protein-coupled receptor (GPCR) signaling [32-34]. Activated Ras is also known to stimulate PI3Ks by directly binding to the catalytic subunit p110 [35]. There is strong evidence for interaction and activation of p110γ by Ras [36]. Ras has also been shown to play a role in activating p110α and p110δ [36]. Both the regulatory and the catalytic class I_A PI3K subunits in human are encoded by three genes respectively, but due to alternate splicing the three genes encoding the regulatory subunits can give rise to five different subunits [37-40]. Even though there are preferences, each p85 can form heterodimers with any of the catalytic p110 isoforms, although the exact role of the different p85 splicing variants is not well understood [41]. Together with the catalytic

Box 1. Classification and domain structure of PI3K family members

A. Classification of PI3K family members

	Isoforms	Gene name	Substrate speifity	Regulator	Cellular function	Expression
Class I_A	**Regulatory**					
	p85α, p55α, p50α	*PIK3R1*	PtdIns	RTKs	-proliferation	ubiquitous
	p85 β	*PIK3R2*	PtdIns(4)P	GPCRs (p110β)	-metabolism	
	p55γ	*PIK3R3*	PtdIns(4,5)P_2	Ras	-migration	
					-survival	
	Catalytic					
	p110α	*PIK3CA*			-immunity	p110δ mainly in
	p110β	*PIK3CB*			(p110δ)	leukocytes
	p110δ	*PIK3CD*				
Class I_B	**Regulatory**					
	p101	*PIK3R5*	PtdIns	GPCRs	-inflammation	mainly in
	p84/p87	*PIK3R6*	PtdIns(4)P	Ras	-platelet	leukocytes
			PtdIns(4,5)P_2		aggregation	
	Catalytic				-immunity	
	p110γ	*PIK3CG*				
Class II	**Catalytic**					
	PI3KC2α,	*PIK3C2A*	PtdIns	RTKs	-vesicular	Broad expression,
	PI3KC2β	*PIK3C2B*	PtdIns(4)P	Cytokine receptors	transport	but not ubiquitous
	PI3KC2γ	*PIK3C2G*		Integrins	-cell migration	
				GPCRs (LPA)	-chemotaxis	PI3KC2γ mainly
						in liver
Class III	**Regulatory**					
	Vps15/p150	*PIK3R4*	PtdIns	GPCRs	-autophagy	ubiquitous
				amino acids, glucose	-vesicular	
	Catalytic	*PIK3C3*			transport	
	Vps34					

B..Domain structures of PI3K family



Box 1. A. Classification of PI3K family members. The PI3K family of enzymes comprises eight catalytic isoforms divided into three classes according to their structural similarities and substrate specificity. **B.** Domain structures of the PI3K family. Class I_A regulatory subunit consists of a p110-binding domain, two SH2 domains, which are responsible for binding tyrosine-phosphorylated residues on proteins, and for the p85α and p85β isoforms, additionally a SH3 domain and a BCR homology (BH) domain flanked by two proline rich regions (P). The shorter splicing variants of p85α lack the N-terminal BH- and SH3 domain. Class I_A catalytic subunit consists of the PI3K core structure containing a kinase and helical domain, followed by a C2 domain. Additionally, the catalytic subunits have a p85 binding domain in the N-terminal for interaction with the p85 regulatory subunit, and a Ras binding domain. The catalytic p101γ isoform of class I_B has the same domain structure as class I_A apart from the lack of p85 binding domain. Class I_B regulatory isoforms, p101 and p87 do not have any homology to other proteins and this far, none of their domains have been identified. Class II PI3Ks comprises only three catalytic isoforms and are characterized by an additional C2 and PX domain in the C-terminal and a proline rich region in the N-terminal, which differs between the three isoforms. The catalytic subunit of class III PI3Ks, Vps34, consists of the PI3K core structure with a helical and a catalytic domain as well as the N-terminal C2 domain. The regulatory isoform, Vps15 (also known as p150) contain a kinase domain, which is thought to be inactive, and several WD40 repeats and HEAT domains.

isoforms, these five regulatory isoforms give rise to fifteen possible p85-p110 combinations in mammals [41].

The PI3K heterodimers bind to activated RTKs via interaction of the Src homology-2 (SH2) domains of the p85 subunit to specific phosphotyrosine residues in the cytoplasmic domains of RTKs [28, 41-45]. The p85 subunit furthermore contains two proline-rich sequences in its N-terminal region, which can bind to Src homology-3 (SH3) domains, such as those present in Src family kinases (Box **1**)

[46, 47]. The p85 regulatory subunit releases its inhibition on the p110 catalytic subunit upon binding of the heterodimer to RTKs. As a consequence of its relocation to the plasma membrane the heterodimer becomes activated. The PI3Ks becomes activated by at least three distinct mechanisms: I) close proximity to the lipid substrates, II) the loss of p85 subunits regulatory effect caused by the SH2-phosphotyrosine docking to RTKs [30, 48], and III) activation through the interaction with Ras GTPase.

The promoter of p110α was recently shown to be under positive control of Forkhead box 03a (FOXO3a) [49] and Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NF-kB) [50] and to be negatively regulated by p53 [51]. Since PI3K signaling inhibits FOXO3a, p110α is able to negatively regulate its own gene expression [52]. In addition to lipid kinase activity, the class I PI3K catalytic isoforms were shown to possess a protein kinase activity, which can phosphorylate the p85 adaptor subunit [53].

The p110α and p110β isoforms are broadly expressed throughout the body. The p110α was recently reported to be enriched in non-proliferating tumor tissue of ovarian cancer *in vivo*, even though it is known to play a role in cellular proliferation [50]. On the other hand, fully differentiated, non-proliferative healthy tissue has been reported to be enriched in the regulatory subunits p85α, p55α and p50α of class I_A [54]. p110δ, which is involved in immune functions and inflammation, is predominantly expressed in leukocytes [55-57]. However, it was unexpectedly also found expressed in the developing nervous system [58].

2.2. Class I_B PI3K Isoforms

Class I_B consists of the regulatory subunit p101, a p101 homolog p84 (also called $p87^{PIKAP}$) and the catalytic subunit p110γ [59-62], which like class I_A form heterodimers. Since p110γ does not bind any SH2 domain-containing p85 regulatory subunit it is not regulated by RTKs. Instead this class is exclusively activated by GPCRs, through direct interaction with the Gβγ subunit [63]. Gβγ recruits p110γ from the cytosol to the membrane by interaction with its p101 or p84 subunit [64]. Accordingly, p101 was found to be required for G protein-mediated activation of p110γin living cells [64]. Studies have confirmed a direct interaction between Gβγ and the N- or C-terminal of p110γ [65]. However, for efficient stimulated phosphorylation of PtdIns(4,5)P_2 *in vitro*, the binding of the regulatory p101 or p84 subunits are required [60, 66, 67]. Ras-GTPases can also regulate p110γ. Ras activates p110γ at the level of the membrane, by allosteric modulation and/or reorientation of the p110γ, implying that Ras can activate p110γ without its membrane translocation [68].

The class I_B PI3K pathway plays an important role in immune cells, such as mast cells, dendritic cells, B and T lymphocytes, and also in platelets, the endothelium and cardiac tissue (discussed in section 3). p110γ is highly enriched in leukocytes, whereas the two regulatory subunits, p101 and p84, are accordingly found preferently expressed in haematopoetic tissue [60].

2.3. Class II PI3K Isoforms

The class II of PI3K consists of three distinct catalytic subunits (PI3KC2α, PI3KC2β, PI3KC2γ). This class of PI3Ks was first identified in *Drosophila* and mammalian cells by sequence similarities to the other PI3Ks, and is characterized by an additional C-terminal C2 domain [69, 70]. Class II isoforms do not bind any regulatory subunit as do class I and III, but the PI3KC2β isoform has been shown to bind to activated EGFR via an interaction with the growth factor receptor-bound protein 2 (Grb2) through its proline-rich motifs at the N-terminus [71]. Upon binding to the EGFR, the enzyme becomes activated. The three catalytic subunits of class II have a substrate specificity restricted to PtdInsP and PtdIns(4)P *in vitro* and can be activated by RTKs, cytokine receptors, integrins and lysophosphatidic acid (LPA) [69, 70, 72, 73].

Class II members have been identified as playing a role in diverse cellular functions, but there are still few reports [73-76]. The PI3KC2α isoform has been reported to play a role in clathrin-mediated vesicular trafficking [77, 78]. Moreover, LPA has been shown to stimulate the production of PtdIns(3)P through activation of PI3KC2β [73], which has been linked to cell migration in human cancer cell lines [73]. Increased expression of class II PI3KC2β stimulated Rac activity in A-431 epidermoid carcinoma cells, resulting in enhanced membrane ruffling and migration speed of the cells [74]. Furthermore, PI3KC2β-overexpressing cells were protected from anoikis and displayed enhanced proliferation, independently of Rac function [74]. A recent study has shown PI3KC2γ to be involved in the regulation of stromal cell-derived factor α (SDF1α)-stimulated chemotaxis [79]. Yu *et al.* [79] suggested that down-regulation of PI3KC2γ might be the mechanism by which Bcr-Abl induces abnormal homing of leukemic cells.

2.4. Class III PI3K

The class III of PI3K is composed of a regulatory subunit (Vps15, also called p150) and a catalytic subunit (Vps34). The class III catalytic subunit was first described in *Saccharomyces cervesiae* where it was identified as a component of the vacuolar protein sorting system, and has since then been documented in all investigated eukaryotes. The human Vps34 was reported to associate with the trans-Golgi network, a key site for the formation of transport vesicles destined for different intracellular compartments [80]. Additionally, Vps34 and Vps15 are required for the induction of autophagy [81-85]. It was shown that Beclin co-immunoprecipitates with Vps34, which is also required for autophagy suggesting that Beclin is a component of the Vps34 complex [86]. Similary to class I PI3Ks, Vps34 might be important for controlling cell growth, through the regulation of the mTOR/S6K1 pathway, which regulate protein synthesis in response to amino-acid availability [26, 81, 82].

The lack of potent inhibitors targeting Vps34, resulting from a smaller adenosine triphosphate (ATP)-binding pocket compared to class I PI3K, has for a long time limited the studies of its function [87]. 3-methyladenine (3-MA) is today

most effective inhibitor of Vps34, but it has to be used at very high concentrations (10mM) in cells to inhibit autophagy [88]. However, the crystal structure of Vps34 was recently reported, giving important information which now may be used for the designing novel Vps34-selective inhibitors. There are yet no studies performed with Vps34 knockout mouse models, but genetic studies of mutated Vps34 and Vps15 genes in *Drosophila* and *C.elegans* have given a greater insight into the function of class III PI3K isoforms. Introduction of the mutated gene *irdl*, (the *Drosphila* homologue of Vps34-related kinase Vps15), which additionally is presumed to also inhibit dVps34, resulted in a phenotype of starvation-induced activation of antimicrobial peptides, which suggests a role of Vps34 in the innate immune system [89]. In addition, genetic approaches targeting *let-512* (Vps34 gene homologue in *C.elegans*) induced embryonic lethality and impaired membrane transport between the outer nuclear membrane and the periphery of the cell [90].

The information about the involvement of class III PI3Ks in human diseases is rather limited. So far there are only two reports linking mutations in the Vps34 promotor region to schizophrenia [91, 92]. However, due to the importance of Vps34 for mTOR signaling [81, 82] inhibiting Vps34 may become useful in the treatment of insulin resistance in obesity (according to [93]). Furthermore, in the context of tumor suppression an up-regulation of Vps34 would lead to enhanced autophagy [94], which in turn also is needed for the clearance of pathological protein aggregations in diverse neurodegenerative diseases. Therefore, pharmacological up-regulation of Vps34 could possibly become a new therapeutic approach for treating these diseases.

3. MOUSE MODELS

Mouse knock-out and knock-in models have greatly contributed to our understanding of the function of class I PI3Ks in normal tissue and cells, and additionally helped confirming their role in diseases [95]. Furthermore, these mouse models, in combination with gene targeting methods, have been useful and facilitated the design of inhibitors targeting the PI3K/Akt pathway for treatment of diseases. But since the different isoforms seem to have the potential to compensate for each other, there have been difficulties in elucidating the exact function of the single isoforms of the PI3K family. Additionally, homozygous knock-outs of the majority of class I catalytic subunits were found to be lethal, a fact which further hindered the investigations of their functional relevance [96].

Homozygous knock-out of either *PIK3CA* or *PIK3CB* yielded an early embryonic lethal phenotype [97, 98], while mice deficient in *PIK3CG* or *PIK3CD* isoform survived to adulthood (Table **1**) [55, 99-101]. However, double heterozygous mice of $PIK3CA^{(+/-)}$ $PIK3CB^{(+/-)}$ survived, but with impaired insulin responses [102]. Furthermore, knock-in studies resulting in kinase-dead p110α phenotype gave similar result, suggesting an important kinase-dependent role for p110α in insulin signaling [102, 103]. These findings have subsequently been confirmed with pharmacological inhibitor studies [103, 104].

While p110α was found to play an important role in short-term insulin receptor signaling, p110β was described to be important to sustain long-term insulin signaling [105]. It was reported that mice carrying a kinase-dead p110β mutant survive to adulthood, but develop mild insulin resistance at

Table 1. Mouse Models of PI3K Signaling Pathway

Target	Viability	Phenotype	Alteration in PI3K Subunit Expression	Refs.
p110α	-/- : embryonic lethal $^{*KD}/^{KD}$: viable $^{KD}/^{+}$: viable	- impaired insulin signaling decreased growth factor/metabolic signaling	No alterations in p110α, p110β, p110δ or p85	[98] [103]
p110β	-/- : embryonic lethal $^{KD}/^{KD}$: viable	- retarded growth, Impaired insulin signaling		[97] [105]
p110δ	-/- : viable $^{KD}/^{KD}$: viable	Impaired B-and T-cell function, attenuated immunresponse Impaired B-and T-cell antigen receptor signaling, attenuated immune response, developed inflammatory bowel disease		[95, 101, 107] [101]
p110γ	-/- : viable	impaired T-cell survival, increased NF-κB activation		[113, 115]
p85α	-/- : viable +/- : viable	Increased insulin signalling, impaired B-cell development, no T-cell independent antibody production Increased Akt activity, increased apoptosis	↑ p55α ↑ p50α	[117-120, 122, 128, 129] [120]
p85α/p55α/p50α pan	-/- : perinatal lethal +/- : viable	Hypoglycemia, hypoinsulinaemia Increased insulin sensitivity, reduced number of mature B-cells, decreased serum immunoglobin	↑p85β ↓p110α ↓p110β ↑p85β, p110α and p110β not affected	[118-120, 122] [120, 130]
p55α/p50α	-/- : viable	Increased insulin sensitivity	In muscles ↓p85α	[131]
p85β	-/- : viable	Increased insulin sensitivity	No alteration reported	[122]

*KD - kinase-dead.

the age of 6 months, in addition to suffering from a retarded growth rate [105]. Furthermore, mice carrying a conditional knock-out *PIK3CB* allele, with liver-specific deletion, showed higher levels of insulin in the blood, compared to the wild type animals, but without significant changes in Akt phosphorylation [105]. Additionally, mouse embryonic fibroblasts (MEFs) lacking p110β proliferated slower than wild type cells and showed impaired insulin sensitivity, without changes in Akt phosphorylation status [105, 106]. The catalytic function of p110β was not needed for phosphorylation of Akt upon stimulation with EGF, IGF-1 or PDGF, but its function was however required for activation of Akt upon LPA and sphingosine-1-phosphate (S1P) stimulation [105]. Taken together, these results show that p110β posseses both a kinase- dependent and a kinase-independent function. Low levels of p110β led to embryonic lethality, while the presence of catalytically inactive p110β was sufficient to proceed through development and led to survival into adulthood [105]. The p110β catalytic function is required for development, while insulin signaling is a kinase-independent function of p110β. Additionally, the abovementioned results provide an evidence for the involvement of the p110β isoform in GPCR signaling.

Mice deficient in p110δ were viable, but had severely impaired T and B cell function [101, 107]. In mice expressing a catalytically inactive form of p110δ ($p110\delta^{(D910A)}$), the antigen receptor signaling in B and T cells was impaired and immune responses *in vivo* were attenuated [101]. Resting B cells with catalytically inactive p110δ failed to enter the cell cycle and p110δ activity was also critical for interleukin 4 (IL-4)-signaling and protection from apoptosis [108]. Inactivation of p110δ significantly impaired the ability of B cells to activate T cells in a BCR-mediated antigen uptake and presentation model [109]. Additionally, the transgenic mice developed inflammatory bowel diseases [101]. These results revealed a selective role for p110δ in immunity [101, 110, 111].

Mice deficient in both *PIK3CG* and *PIK3CD* were generated and shown to have a profound block in T cell development that occurs at the β-selection checkpoint [112]. Mice lacking the GPCR-coupled p110γ were viable and had fully differentiated neutrophils and macrophages [113, 114]. Chemoattractant-stimulated *PIK3CG* $^{(-/-)}$ neutrophils failed to produce PtdIns(3,4,5)P_3, did not activate Akt, and displayed impaired respiratory burst and motility [55, 113, 115, 116].

Deletion of the gene encoding the regulatory subunit p85 alone was shown to change the expression and activity of other regulatory and catalytic subunits of class I PI3K (Table **1**). Mice deficient in p85α showed increased insulin signaling and displayed up-regulation of the p55α and p50α regulatory subunit [117]. Total knock-out of *PIK3R1* (p85α and its splicing variants p55α, p50α) resulted in prenatal lethality [118, 119]. The mice died due to the reduction in class I_A PI3K catalytic function which led to abnormalities in multiple tissues, such as extensive hepatocyte necrosis, hypoglycemia and hypoinsulinaemia [119]. The animals furthermore displayed reduced numbers of peripheral mature B cells and decreased serum immunoglobulins [118]. Heterozygous disruption of *PIK3R1* resulted in increased Akt activity and decreased apoptosis by IGF-1 through up-regulated PtdIns(3,4,5)P_3 production [120]. Complete depletion of p85α, on the other hand, resulted in significantly increased apoptosis due to reduced PI3K-dependent signaling [120]. These findings revealed that p55α and/or p50α are required for survival, but not for development of hypoglycaemia in mice lacking p85α [119].

As with the homozogous knock-out of p85α, homozygous knock-out of p85β yieled mice with hypoinsulinemia, hypoglycemia, and improved insulin sensitivity [121]. $p85\beta^{(-/-)}$ cells however, exhibited significantly increased insulin-induced Akt activation, despite the reduction in regulatory subunits, leading to increased anti-apoptotic signaling [122]. Thus, p85α and p85β modulate PI3K-dependent signaling by multiple mechanisms and transmit signals independently of PI3K activation [122]. The observation that depletion of the regulatory subunits can enhance insulin signaling has been explained in two ways: i) deletion of monomeric p85 gives the chance for p110/p85 heterodimers to signal without competition, ii) p85 monomers are unstable [122]. Deletion of p110α or p110β reduced the overall p85 levels, and additionally it was shown that introduction monomeric p85α into adipocytes lacking p85α, p55α and p50α was not sufficient to restore PI3K activity [102, 122-124]. These results indicate that, in addition to their roles in recruiting the catalytic subunit of PI3K to the insulin receptor substrate proteins, both p85α and p85β play negative roles in insulin signaling [121].

p101-null mouse neutrophils demonstrated impaired GPCR ligand-triggered induction of chemotactic responses [125]. However, even though both the induction of neutrophil NADPH oxidase and the GPCR-ligand induction are dependent on p110γ, only GPCR induction was affected by the depletion of p101 [113, 125].

The investigation of the function of PI3K family members has additionally been carried out by constructing mouse strains with a constitutively activated PI3K pathway. Overexpression of mutated proteins or other alterations in the signaling pathway have provided evidence for the importance of genetic alterations for the development of different cancer phenotypes. PI3K/Akt signaling can be triggered by activating mutations, amplifications or deletion of *PTEN*. However, overexpression of any of the PI3K pathway components alone is not enough to cause cancer. It has to be expressed in combination with other oncoproteins from a second pathway. For example, mice expressing the activated p85 allele, $p65^{pi3k}$ were reported to develop lymphoproliferative disorders, but did not develop lymphoma until crossed with p53-/- mice [126]. Additionally, mouse brain progenitor cells co-transfected with activated alleles of Ras and Akt developed glioblastoma, whereas single transfection of either gene alone did not induce tumorigenesis [127].

4. PI3K IN HUMAN DISEASES

Studies of mouse models have been of great importance for elucidating the biological functions of the PI3K family, and have furthermore given valuable information about the involvement of these enzymes in human diseases. Most studies have been focused on PI3Ks connection to cancer, but the list of human diseases in which PI3K signaling is involved is much longer than that. In the next section, we will describe human diseases with existing mouse models,

showing the involvement of PI3Ks and their central role in disease evolvement and progression.

4.1. PI3K Deregulation in Cancer

Tumorigenesis is a multistep process involving genetic alterations in a large number of genes controlling processes such as cell proliferation, growth, apoptosis, migration and metabolism. It is therefore not surprising that PI3K family members are frequently mutated in cancer at different stages of tumor progression starting at the initiation phase up to the stage of metastasis. The PI3K/Akt pathway can be activated through various mechanisms, including gene amplification, mutations and loss-of-function of the PI3K antagonist *PTEN*. The observation that the tumor suppressor PTEN functions as an antagonist of PI3K gave the first firm link between PI3K activity and cancer [132]. In addition, PI3Ks were previously found associated with the activity of the Rous sarcoma $pp60^{v\text{-}src}$ protein and the polyoma middle T antigen oncogenes [133-135], which further supported the importance of the PI3K family in tumorigenesis.

The PI3K pathway is, after the p53 tumor suppressor pathway, the most highly mutated pathway in human cancer [136]. Most mutations were reported in *PIK3CA* and *PTEN* so far. Somatic mutations in *PIK3CA*, encoding the class I_A p110α, and *PIK3RI*, encoding p85, promote the activation of the PI3K/Akt pathway [137-140]. Activating mutations in *PIK3CA* have been reported in many cancer types including breast, colon, endometrial, glioblastoma, ovarian and hepatocellular cancers (references see Table **2**). Additionally, *PIK3CA* gene amplification is most frequently encountered in cervical, lung, gastric, ovarian and head and neck cancers [141, 143, 145, 148, 177]. Interestingly, most of the mutations in *PIK3CA* are clustered in two "hot spot" regions in exon 9 and 20 which encode the helical and kinase domain respectively (Box **1**) [139, 147]. Mutations in the kinase domain result in enhanced enzymatic activity, while mutations in the helical domain de-repress the inhibitory effect of p85 on the p110α catalytic subunit [149, 150]. In addition to the "hot spot" mutations, up to 100 rare *PIK3CA* mutations have been reported in various cancers [151]. Fifteen of these rare mutations were further characterized and all but one showed increased oncogenic function [152].

Although activating mutations in *PIK3CA* frequently occur, there is so far no evidence of activating oncogenic mutations in the other class I_A or I_B catalytic subunits. Overexpression of p110β was reported in several tumor types [153, 154], as well as rare cases of somatic single-residue substitution in p110β and in p110γ, but the effect of these substitutions remains to be studied [155]. However, over-expression of wild-type p110β, p110δ or p110γ was found to be transforming in cell culture unlike the p110α isoform [31]. A recent study showed an important role for p110β in *PTEN*-deficient tumors. *PIK3CB* depletion in a mouse model of prostate cancer induced by *PTEN* knock-out, led to decreased PI3K signaling and prevented prostate tumorigenesis [106]. Double knock-down of *PTEN* and *PIK3CA* on the other hand, had no significant effect on tumorigenesis, suggesting that it could be worth considering treating *PTEN*-deficient tumors by targeting the p110β isoform even if it is a *PIK3CA*-mutation driven tumour (suggested by [155]). Furthermore, studies in mice using an HER2/ErbB-2 triggered breast cancer model showed that the expression of kinase-dead p110β delayed tumor development compared to control mice [105]. This result was confirmed by inhibitor studies, demonstrating the involvement of p110β catalytic function in HER2-driven tumors [105].

p110δ expression is normally restricted to leukocytes, but there are reports showing that the p110δ isoform is over-

Table 2. Genetic Alterations of the PI3K Pathway

Genetic Mutations	Affected PI3K Isoforms	Most Common Cancer Types	Refs.
PIK3CA	p110α	Breast, colon, gastric,, glioma, endometrial carcinoma, hepatocellular carcinoma	[141, 142, 144, 146-148, 159]
PIK3R1	p85α	Colon, ovarian, breast cancer	[137, 160]
PIK3R5	p101	Colon cancer	[160]
PIK3R4	Vps15/p150	Breast cancer	[160]
AKT1 (E17K)		Breast, ovarian, colorectal, sqamous cell lung carcinoma, acute leukemia	[163, 164, 170]
AKT2		Colorectal, gastric, lung	[167, 176]
AKT3		Melanoma	[168]
PTEN		Glioblastoma, melanoma, breast, prostate, ovarian, thyroid, lymphoma, hepatocellular carcinoma endometrial carcinoma, renal-cell carcinoma	[13-25]
Genetic Amplification	**Affected PI3K Isoforms**	**Most Common Cancer Types**	**Refs.**
PIK3CA	p110α	Cervical, ovarian, lung, thyroid, gastric, head and neck sqamous cell carcinoma	[141, 143, 145, 177-179]
AKT1		Gastric cancer	[169]
AKT2		Head and neck, pancreatic, ovary, breast cancer	[169, 170]

expressed in colon and bladder cancer [153], as well as in neuroblastoma [156], and acute myeloid leukemia [157, 158]. Additionally, elevated mRNA levels of p110δ have been reported in glioblastoma [159]. Inhibitor studies have furthermore shown p110δ to be critical for the proliferation of acute myeloid leukemia blast cells [158].

The class I_A regulatory subunit p85 (*PIK3R1*) was reported to be mutated with a low frequency in breast, colon, and ovarian cancer [137, 160]. Alterations in *PIK3R1* are known to cause impaired binding of the p85α subunit to the p110 isoforms, caused by alterations in the SH2 domain of p85α. This mutation leads to loss of p85α negative regulation of the p110 isoform catalytic activity [149]. As a conesquence, the p110 isoform is now able to initiate a downstream cascade of antiapoptopic and cell growth signaling through increased phosphorylation of Akt [161].

The familiy of Akt isoforms (Akt1, Akt2 and Akt3) were also found to be mutated in human cancers. Somatic mutations in *AKT1* were discovered in breast, colorectal, ovarian and lung cancers as well as in leukemia [162-164, 170]. The most common mutation (E17K) alters the lipid binding site of Akt1 (PH domain), causing constant membrane localization, even in the absence of PtdIns(3,4,5)P_3 [162]. On the other hand, there are a few reports available which demonstrate the lack of involvement of *AKT1* PH-domain mutation in the development of hematologic malignancies, such as acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), prolymphocytic leukaemia (PLL) [165, 166]. Somatic mutations of *AKT2* and *AKT3* were also reported in different tumor types [167, 168]. *AKT1* and *AKT2* are furthermore often found amplified in human cancer (Table **2**) [169, 170].

One of the most common genetic alterations in the PI3K pathway is inactivation of the *PTEN* gene. The expression of *PTEN* can be altered through several mechanisms: loss of heterozygoty (LOH), point mutations, homozygous deletion and epigenetic silencing via promoter methylation. Alterations in *PTEN* expression are responsible for clinical syndromes such as Cowden's disease, Lhermitte-Doclos disease, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome and Proteus-like syndrome [174], with predisposition to cancer. Loss-of-function mutations in the *PTEN* gene are also extremely common in giloblastoma, melanoma, prostate, breast, ovarian, endometrial, thyroid, lymphoid, and colorectal cancer, and in hepatocellular and renal-cell carcinoma (references see Table **2**).

In normal cells, PI3K signaling is under the regulation of RTKs, but in cancer RTKs are often mutated, amplified or over-expressed, causing constant PI3K signal activation. Therefore, inhibitors targeting the upstream RTKs may represent promising therapeutics for cancer patients whose tumors have a deregulated PI3K/Akt pathway. However, activating mutations downstream of RTKs cause constitutive activation of the pathway and are therefore believed to also cause resistance towards therapies targeting RTKs [175].

4.2. Autoimmune and Inflammatory Diseases

Inflammation is a physiological reaction of an immune system in response to injuries or exposure to pathogens. During this "state of emergency", different kinds of immune cells (leukocytes) are mobilized to overcome the threat for the organism. PI3Ks are known to be expressed in leukocytes and to be involved in the development, migration and function of these cells (reviewed in [110, 180, 181]). Consequently, these enzymes control different kinds of immune responses: the innate response, which induce neutrophils, eosinophils, macrophages and mast cells, and the adaptive response, which engages T- and B-lymphocytes (reviewed in [180, 181]). Since *PIK3CA* and *PIK3CB* mouse knock-out models are lethal at the early embryonic stage, their function in the immune system has not been studied exhaustively. The class I_A p110δ and class I_B p110γ are considered to be key players in inflammation. Loss-of-expression of *PIK3CD* or *PIK3CG* is not lethal, but it causes severe malfunctions in immune responses of animal models [95]. The data derived from animal studies support the model that the p110δ and p110γ isoforms play non-redundant functions in immunity, but also cooperate with each other to regulate immune system responses [95]. Inactivation of p110δ reduced B- and T-cell receptors signaling [101], whereas p110γ-deficient mice, beside impaired T-cells activation, also displayed impaired neutrophil and macrophage migration [113, 115]. It was demonstrated in double mutant p110δ/$\gamma^{-/-}$ mice that the combined functions of these two isoforms are required for critical steps in T-cell development [112]. Moreover, both p110δ and p110γ contribute to PtdIns(3,4,5)P_3 accumulation in tumor necrosis factor α (TNFα)-primed human neutrophils after formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation. p110γ is responsible for a first phase of PtdIns(3,4,5)P_3 synthesis, whereas a second phase depends on p110δ action and requires TNFα-pretreatment [182]. On the other hand, it has been recently shown that p110δ, but not p110γ, mediates optimal IgE/Ag-dependent allergic response *in vivo* [183]. Deregulation of any p110δ and p110γ-driven signaling pathways in inflammation leads to development of various autoimmune, respiratory and cardiovascular diseases.

4.2.1. Autoimmune Diseases

Systemic lupus erythematosus (SLE) is a chronic inflammatory disorder occuring mostly in females from African or Asian countries. The risk of SLE development strongly depends on genetic predispositions which drive the loss of the tolerance of the immune system to nuclear antigens, an increase in B-cell activation and dysregulate T-cells (reviewed in [184, 185]). When the $CD4^+$ memory T cells are deregulated, B-cell activation is prompted, which in turn leads to hypergammaglobulinemia and elevated production of DNA-specific autoantibodies. These anti-DNA antibodies contribute to tissue damage by binding to native double-stranded DNA of host cells, especially kidney cells, where they form deposits, settle and promote local inflammation followed by glomerulonephritis and, in the worst case, renal failure (reviewed in [184, 186]). p110γ was shown to play an important role in SLE progression. Pharmacological inhibition of this isoform with the AS605240 compound was investigated in the SLE-susceptible MRL-*lpr* mouse model exhibiting increased activation of $CD4^+$ T cells and PI3K/Akt pathway. Inhibition of p110γ extended the lifespan of the animals, reduced anti-DNA antibody levels and the absolute number of pathogenic $CD4^+$ memory cells, and

prevented glomerulonephritis and proteinuria progression [187]. A similar decline in SLE symptoms was obtained in p110γ-deficient mice constitutively expressing $p65^{PI3K}$, which is a truncated form of p85 regulatory subunit. When $p65^{PI3K}$ is expressed alone it causes up-regulation of the PI3K pathway and induces SLE development [126, 188]. Moreover, inactivation of *PTEN* also triggers the SLE phenotype, further emphasizing an important role of PI3Ks in progression of this disease [126].

Rheumatoid arthritis (RA) is another chronic inflammatory disease that affects synovial joints and results in serious problems with mobility (explained in [189, 190]). Degradation of cartilage and bone in the joints is driven by infiltration of macrophages, neutrophils, synovial fibroblasts, as well as B- and T-cells, but the exact causes and pathogenesis of the disease are not completely understood (reviewed in [189, 190]). Since it was shown that p110γ mediates leukocytes migration [113, 115], it was suggested that the enzyme may be involved in RA pathogenesis. Passive type II collagen-specific antibody-induced arthritis (αCII-IA) mouse model (T- and B-cells independent) lacking p110γ ($p110\gamma^{-/-}$) showed defective neutrophil migration when compared to $p110\gamma^{+/+}$ mice and, as a consequence, did not developed severe RA symptoms [191]. Pharmacological blockade of p110γ with AS-605240 inhibitor in the wild type αCII-IA model, and in the distinct collagen-induced arthritis (CIA) mouse model (T- and B-cells dependent) also inhibited neutrophil migration and accumulation in the joints, preventing disease progression [191]. p110γ-deficient mice were therefore protected from RA dvelopment, independently of the model used. Recently, an alternative human tumor necrosis factor (TNF)-dependent transgenic mouse model (hTNFtg) for studying the chronic, destructive phase of RA was used [192]. p110γ-lacking hTNFtg mice showed less severe inflammatory arthritis. This phenotype did not result from decreased invasion of immune cells, but was rather due to impaired expression of matrix metalloproteinases in fibroblasts and chondrocytes, driven by reduced phosphorylation of Akt and extracellular signal-regulated kinase (ERK). As a consequence, cartilage damage and joint destruction was stopped confirming the therapeutic potential of p110γ inhibition in RA [192]. Interestingly, a similar decrease in joint and cartilage erosion was obtained *in vivo* as a result of *PIK3CD* gene deletion or selective, pharmacological inhibition of p110δ enzymatic activity obtained with IC87114 [193]. Importantly, inactivation of both p110δ and p110γ enzymes *in vivo* through IC87114 administration to $p110\gamma^{-/-}$ animals, almost completely abolished joint injury caused by leukotriene B4 (LTB4)-mediated neutrophil influx into inflamed tissue. These findings indicate that in response to the chemoattractant LTB4, which stimulates autoantibody-mediated arthritis in mice, p110δ collaborates with p110γ to induce maximal neutrophil migration [193]. Taken together, targeting p110δ and p110γ can result in significant therapeutical benefits in blocking RA progression.

4.2.2. Respiratory Diseases

Allergic asthma is a chronic disease of the respiratory system characterized by airway hyper-responsiveness (AHR), inflammation and reversible obstruction, as well as mucus secretion in response to allergen [194]. The pathogenesis of asthma strongly depends on elevated levels of IgE antibodies and activation of immune cells such as T helper type 2 (T_h2) cells, mast cells, neutrophils, B cells and eosinophils, which produce a number of cytokines, chemokines, adhesion molecules, growth factors and enzymes contributing to disease progression (reviewed in [194]). PI3Ks are part of the complex signaling network involved in asthma development (reviewed in [195, 196]). Genetic inactivation of p110δ in ovalbumin (OVA)-challenged mice decreased T_h2 cytokine-dependent airway inflammation, mucus production and reduced eosinophil recruitment [197]. Similar findings were reported with the use of a pharmacological approach, the p110δ-selective inhibitor IC-87114, in the mouse asthma model sensitized with OVA [198]. Furthermore, depletion of p110δ protected mice against anaphylactic responses [199]. For these reasons, p110δ was proposed as a new target for the treatment of allergy and mast-cell-related pathologies. Additionally, two separate studies reported that, in contrast to wild type animals, OVA-induced p110γ-deficient asthma mouse models stimulated with the allergen exhibited a significant reduction in T_h2 cytokine production, decreased airway remodelling, inflammation and hyper-responsiveness [200, 201]. Taken together, these findings indicate a role for p110δ and p110γ in the development of allergic asthma and emphasize their potential as targets for therapeutical intervention.

Chronic obstructive pulmonary disease (COPD) is another common airway dysfunction which is characterized by airflow limitation, but in contrast to asthma, is poorly reversible and progressive [202]. Administration of a p110δ/p110γ selective pharmacological inhibitor TG100-115 to mouse models of COPD reduced lipopolysaccharide (LPS) and smoke-induced pulmonary inflammation, suggesting the therapeutic potential of p110δ and p110γ inhibitors for the COPD treatment [203].

In addition to all these reports documenting the therapeutic potential of the PI3K isoforms in diverse autoimmune human disorders, we can add that inflammation, which is partially driven by the PI3K/Akt pathway, may also lead to the development of cancer [204].

4.3. Cardiovascular Diseases (CVDs)

The term CVDs encompasses all disorders of the heart and blood vessels, including those arising on the basis of inflammatory defects such as atherosclerosis.

Atherosclerosis is a chronic inflammatory disease affecting large arteries, culminating with cardiovascular disorders such as myocardial infarction and heart failure, stroke, renal damage or acute limb ischaemia (reviewed in [205] and [206]). Inflammation processes are involved in all phases of atherosclerosis, starting from atherosclerotic plaque formation, up to its disruption. All types of immune cells migrate to atherosclerotic plaques, but the crucial step for the onset of the disease, the transformation of oxidized low density lipoprotein (Ox-LDL) to foam cells, is driven by macrophages (reviewed in [205, 207]). It is known that Ox-LDL triggers PI3K signaling in macrophages/foam cells, which produce growth factors and cytokines for PI3K pathway stimulation (reviewed in [205, 207, 208]). p110γ plays an important role in the activation of macrophages induced by Ox-LDL, angiotensin II (AngII) and chemokines [209].

ApoE$^{-/-}$ mouse models of acute atherosclerosis lacking p110γ (p110γ$^{-/-}$) demonstrated a reduced size of the plaque in comprison to ApoE$^{-/-}$p110γ$^{+/+}$ or ApoE$^{-/-}$p110γ$^{+/-}$ mice [209]. Furthermore, p110γ expression in atherosclerotic lesions was shown to be elevated in murine model, whereas loss of the isoform impaired the macrophage and T-cell infiltration, which was associated with increased plaque stabilization [210]. Taken together, these results indicate that p110γ can serve as a potential therapeutic target for atherosclerosis treatment.

When not cured, atherosclerosis usually causes an interruption in the blood and oxygen supply to the heart, which leads to cardiomyocyte death and results in *myocardial infarction (MI)* (heart attack). Following cardiomyocyte death an immune response is promoted to activate reparation processes of damaged tissue (reviewed elsewhere: [211] and [212]). The contribution of p110δ and p110γ to the inflammation process after ischemic damage was confirmed in pharmacological inhibition studies with the use of a dual p110δ/p110γ inhibitor TG100-115 (described later in section 5.2) [213].

All members of class I PI3Ks, comprising of p110α, p110β, p110δ and p110γ are expressed in the heart, where they mediate distinct cellular functions such as cell survival, metabolism, hypertrophy, contractility and mechanotransduction. p110α and p110γ activation in the heart is the most intensively studied. Together with the PTEN phosphatase, which antagonizes PI3K action, the enzymes are expressed in the cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells (VSMCs) (reviewed in [214]). Thus, in concert with PTEN, ubiquitously expressed PI3K isoforms are involved in both physiological and pathological processes occuring in the heart (reviewed in [214-216]). While p110α mainly regulates heart size (hypertrophy), p110γ influences heart contractility [217]. Constitutive activation of p110α in mice resulted in the development of larger hearts due to an increase in myocyte size, whereas dominant negative mutants developed smaller hearts consisting of smaller myocytes [218]. Analogous results were also obtained by constitutive expression of Akt or inactivation of *PTEN*, which caused pronounced elevation of Akt and S6K phosphorylation levels [217, 219]. Rapamycin treatment of mice expressing constitutively active Akt caused a reduction in heart size, demonstrating that mTOR and/or its effectors are involved in controlling physiological heart hypertrophy [219]. The fact that cardiac deletion of *PIK3R1* encoding regulatory subunits also leads to a decrease in heart size provides a proof-of-concept for the hypothesis that class I_A PI3K, especially p110α, are necessary and sufficient to regulate cardiac hypertrophy [220].

In the physiological context of heart hypertrophy, which is an adaptive process stimulated for example by physical exercise, p110α is activated upon insulin growth factor (IGF-1) binding to RTKs, in particular to the IGF-1R [221]. In contrast, pathological hypertrophy, resulting from pressure overload on cardiac walls (*hypertension*) and showing defective adaptation, involves activation of p110γ downstream of GPCRs [222]. Stimulation of GPCRs with different ligands such as AngII, endothelin-1 (ET-1) hormones and catecholamines induces signal transmission to downstream effectors such as mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) and p38 MAPK (see [223, 224]).

Unlike p110α, p110γ-deficient mice displayed hypercontractility of the heart muscle associated with increased levels of cAMP [217]. Loss of *PTEN* in the heart of mice decreased the organ contractility, whereas targeting of p110γ function in *PTEN*-depleted mice completely reverted this phenotype [217]. Importantly, p110γ was shown to control cAMP levels and heart contractility independently from its enzymatic activity, but dependent on its scaffolding role for phosphodiesterase 3B (PDE3B) [216, 225]. Apart from the scaffolding function of p110γ, the enzyme also utilizes its kinase activity for the regulation of β-adrenergic receptors (β-AR) [226]. By interacting with G protein-coupled receptor kinase-2 (GRK-2), p110γ turns off β_2-AR signaling, downregulates cell surface expression of β_2-AR and promotes receptor internalization, which is associated with the development of *heart failure (HF)* [216, 227, 228]. Accordingly, p110γ is considered to play negative role in heart failure, whereas p110α is associated with a positive function.

In addition to CVDs directly associated with the heart, there are some blood vessel disorders which involve the action of PI3Ks. Platelets express all class I PI3K catalytic isoforms, including p110α, p110β, p110δ, and p110γ, but only the role of p110γ and p110β in platelet function has been studied so far [229]. Low expression levels of p110δ and a lack of information concerning the role of p110α in platelets have caused a lack of interest in the investigation of the function of these two isoforms in the context of blood vessel diseases. In contrast, platelets from p110γ-null mice showed impaired aggregation after stimulation with adenosine diphosphate (ADP) which acts via GPCRs [230, 231]. *Arterial thrombosis* modified 'Folts-type' rat and rabbit models and rat cartoid artery electrolytic injury model treated with a selective TGX-221 inhibitor of p110β exhibited defects in arterial thrombus formation without a corresponding increase in bleeding time [232]. These results suggest that both p110γ and p110β can play an important role in the physiological process of platelet adhesion and aggregation at the sites of vascular injury, as well as in the pathological conditions of thrombus formation. If not cured, arterial thrombosis leads to the development of myocardial infarction and ischemic stroke. The major cause of these disorders is assigned to platelets, which get activated by high shear stress conditions. High shear stress conditions usually occur in the blood vessels after sudden disruption of atherosclerotic plaques which normally stimulate excessive platelet accumulation, and as a consequence, changes in blood flow velocity. Rapid blood flow associated with an increase in high shear forces promotes blood clot (thrombus) growth and diseases progression [229, 233]. PI3Ks act downstream of the main platelet receptors involved in shear-induced platelet activation, such as glycoprotein Ib/V/IX and integrin $\alpha IIb\beta_3$ [234]. Platelets aggregation depends on signals triggered by multiple adhesive ligands (e.g. von Willebrand factor, fibrynogen, collagen, vitronectin and soluble agonists like thrombin or ADP) and transduced to a major platelet integrin $\alpha IIb\beta_3$, which mediates platelet–vessel wall and platelet-platelet adhesive interactions. Maintaining platelet adhesion under rapid flow conditions depends on

integrin $\alpha IIb\beta_3$-driven calcium flux combined with ADP release [235].

4.4. Metabolic Diseases

The development of metabolic disorders such as *obesity* or *diabetes mellitus* results from perturbances in glucose homeostasis, which crucially depends on the action of insulin. The major role of insulin is to stimulate glucose uptake from the blood and its metabolization in peripheral tissues, e.g. muscles [236]. The impaired ability of muscle tissue to respond to changing concentrations of insulin is called insulin resistance. It develops as an effect of abnormalities in insulin signaling and leads to serious health problems, such as hyperglycemia, diabetes mellitus type 2, obesity, cardiovascular disease, hypertension, heart failure, heart attack, stroke, increased incidence of cancer and psychosocial problems [237, 238].

The main signaling event which accounts for insulin resistance involves the translocation of the main glucose transporter (GLUT-4) to the plasma membrane [239]. PI3Ks and their downstream effectors are involved in the insulin signaling cascade controlling GLUT-4 translocation and glycogen synthesis. Binding of insulin to its receptor stimulates the intrinsic kinase activity of the receptor, which results in receptor autophosphorylation and phosphorylation of insulin receptor substrates (IRSs), including IRS1-4, Gab1 and Shc (reviewed in [240]). These in turn, interact with p85 regulatory subunits of PI3Ks, which is followed by PI3K activation and phosphorylation of $PtdIns(4,5)P_2$ at the plasma membrane. $PtdIns(3,4,5)P_3$ act as a docking site for Akt which subsequently is activated by the concerted action of PDK1 and mTORC2. As a result, both atypical protein kinase Cλ and ζ, (PKCλ/ζ) and the Akt substrate of 160 kDA (AS160) get activated. As a consequence, insulin-mediated GLUT-4 translocation from intracellular vesicles to the membrane is stimulated [241, 242]. Deregulation of GLUT-4 translocation prevents the uptake of glucose from the blood and contributes to insulin resistance [243].

Among all class I PI3Ks, the most prominent role in controlling glucose homeostasis was attributed so far to p110α [104]. Pharmacological inhibition of this isoform with a panel of chemically diverse agents (PIK-75, PIK-90, PI-103) blocked insulin-stimulated phosphorylation of Akt in adipocytes and myotubes *in vitro*, as well as glucose uptake *in vivo*. Moreover, mice heterozygous for a kinase-dead p110α mutation displayed impaired insulin, IGF-1 and leptin signaling via IRS proteins. As a consequence, the animals suffered from hyperinsulinaemia, glucose intolerance, hyperphagia and increased adiposity and presented reduced somatic growth [103]. Although p110β is also involved in insulin signaling, its role appears to be less significant. It was demonstrated that p110β associates with IRS-1 and generates a basal pool of $PtdIns(3,4,5)P_3$ which is not sufficient for complete activation of Akt. Instead, this pool of p110β-generated $PtdIns(3,4,5)P_3$ defines the amount of p110α activity required for Akt phosphorylation [104]. This data goes in line with results observed in the liver of *PIK3CB* knockout mice, which showed that decreased insulin sensitivity and glucose metabolism correlate with small effect on Akt phosphorylation in response to insulin [106].

Despite the positive function of the p85 regulatory subunit in the complex with class I PI3K, p85 has been also described to play a negative role in the regulation of insulin responses [102, 121]. Different models to explain this dual function were proposed. One of them suggests that p85 can exist *in vivo* in two forms, as a dimer associated with p110 (PI3K), but also as a monomeric form [102]. While p110-p85 duplexes positively regulate insulin signaling by producing $PtdIns(3,4,5)P_3$ second messengers at the plasma membrane, free p85 acts as a negative regulator. Therefore, insulin sensitivity depends on a balance between the p85 and p110 subunits. When not protected by p110, the monomeric p85 form is much less stable than the complexed form. Therefore, in $p85^{+/-}$ and $p85^{-/-}$ mouse models the balance is shifted towards the p110-p85 complex, which results in increased insulin sensitivity [102, 121]. Interestingly, it was demonstrated that p85α inhibits insulin signaling via positive regulation of PTEN [244]. Liver *PIK3R1* knock-out mice showed enhanced insulin signaling and activation of Akt, although the exact mechanism of p85α-dependent regulation of PTEN activity is not known. Since PTEN protein levels in the liver of *PIK3R1* knock-out mice were not changed, the mechanism of regulation is probably not linked to modulations of PTEN expression [244]. In addition to these observations, polymorphism in the human *PIK3R1* gene encoding p85 regulatory subunit is classified as one of the risk factors for developing type 2 diabetes [245].

5. PI3K ISOFORMS AS TARGETS FOR SPECIFIC SMALL MOLECULE INHIBITORS

5.1. PI3K Inhibitors in Cancer Treatment

The involvement of PI3K family members in many human diseases, the gene mutations of PI3K enzymes in different types of cancer and their contribution to major mechanisms of resistance to anti-tumor therapies have stimulated a great interest in the development of PI3K/Akt pathway-targeting strategies. Uncovering the complicated molecular mechanisms governing human disease progression promoted the development of genetic (e.g. RNAi technology) and biochemical tools (e.g. small molecule inhibitors, monoclonal antibodies) which can disrupt the function of particular PI3Ks in both physiological and pathological states. Small-molecule PI3K inhibitors have turned out to be the most successful technology in preclinical settings so far. Here, we will discuss the most significant advances that have taken place during the last years in the area of PI3K inhibitors, which has progressed dramatically. Small-molecule inhibitors became both powerful chemical tools used in research to understand the biological roles of PI3Ks, and potential drugs to be used in the clinics to treat patients suffering from many diseases, especially from cancer.

The natural compound wortmannin and the synthetic flavone LY294002 (Table **3**), represent the earliest generation of PI3K inhibitors. Due to their significant limitations they have never been employed in the clinics, but their role in shaping our current knowledge about PI3K biology is unquestionable. The lack of selectivity and ability to discriminate between different PI3K isoforms together with high *in vivo* toxicity of both compounds (dermal and liver side effects) created a need for the development of new PI3K

inhibitors [246, 247]. As a result, SF1126 and PI-103 as inhibitors with increased stability and potency were developed.

SF1126 was designed as a derivative of LY294002 (Table **3**) [248]. It is conjugated to an RGD integrin-binding peptide ($\alpha v\beta 3/\alpha 5\beta 1$) which targets the compound to the tumor vasculature and to tumor cells [248, 249]. This potent pan-PI3K prodrug overcomes the poor water solubility, and undesirable toxicity of LY294002, which makes it a good candidate for clinical applications. As LY294002, it inhibits other kinases including mTOR, DNA-dependent protein kinase (DNA-PK), Pim-1 oncogene (PIM1), polo-like kinase 1 (PLK1), and casein kinase 2 (CK2) [250]. Its potent antitumor activity was shown *in vitro* and *in vivo* in various cancer cell lines, as well as glioma, prostate and breast cancer xenografts [249]. The compound turned out to be an effective anti-angiogenic agent in six different xenograft models. Sustained inhibition of tumor growth as well as suppression of Akt and ribosomal S6 protein kinase phosphorylation was observed in SF1126-treated tumors up to several hours after cessation of drug administration [249]. Safety and tolerability of SF1126 in patients with advanced or metastatic tumors is currently being tested in a phase I clinical trial [251]. Initial reports from clinical trials demonstrated good tolerability and activity of the drug, which led to disease stabilization in patients with refractory tumors [248, 250].

PI-103 was developed as a novel, very selective, dual PI3K (class I)/mTOR inhibitor (Table **3**) [252]. The drug showed significant *in vivo* anti-tumor activity with no observable toxicity. It inhibited tumor cell invasion, metastasis and angiogenesis in breast and ovarian cancer xenograft models. Moreover, it induced a proliferative arrest in glioma cells regardless of alterations in the PI3K pathway (*PTEN*, *EGFR*) and inhibited the growth of glioma xenografts [253]. In mouse models of ALL the inhibitor significantly enhanced cell-cycle arrest and death of cancer cells [254]. Furtermore, treatment of *PTEN*-null PC3 prostate and *PIK3CA*-mutated HCT116 colon cancer cells revealed a significant decrease in phosphocholine (PC) and total choline (tCh) levels, the increase of which normally serves as a biomarker of tumor progression [255]. Despite the many advantages of PI-103, this inhibitor could not be used for clinical development mainly due to its limited aqueous solubility and extensive metabolism [252]. These two pharmaceutical properties were substantially improved in the next generation inhibitor, a structural modification of PI-103, termed GDC-0941 (Table **3**) [256, 257]. It is a potent, orally bioavailable, ATP-competitive inhibitor of class I PI3K which exhibits a higher selectivity towards $p110\alpha$ and $p110\delta$ than towards $p110\beta$ and $p110\gamma$, and a much higher activity against class I PI3Ks versus classes II and III (including PI3KC2β and Vps34), as well as DNA-PK and mTOR [256]. Its activity *in vitro* is comparable to that of PI-103. Although GDC-0941 is much less active against mTOR and DNA-PK, it led to an anti-proliferative response and presented an anti-angiogenic potential in human cancer cells [257]. *In vivo* antitumor activity was observed when the compound was administrated orally to *PTEN*-null U87MG glioblastoma, as well as *PTEN*- and *PIK3CA*-mutant IGROV-1 ovarian cancer xenografts in athymic mice. Inhibition of tumor growth, prolonged inhibition of the PI3K pathway, including a significant reduction in Akt, GSK3β and S6K phosphorylation were observed [257]. Recent pharmacogenomic studies aiming at the identification of predictive biomarkers of GDC-0941 response in tumors, reported that breast cancer cell lines and tumor xenografts harboring mutations in *PIK3CA* or *HER2* amplification were much more sensitive to the drug treatment than models without these alterations [258]. Similar susceptibilities were observed when two concomitant abnormalities were present (e.g., *PIK3CA* mutation and *HER2* amplification, *PIK3CA* mutation and *PTEN* loss, or *HER2* amplification and *PTEN* loss). In contrast, not all *PTEN*-null models responded as well as *PIK3CA*- or *HER2*-altered ones, suggesting that other factors are required for GDC-0941 sensitization. In addition, pS6K1, pS6, and p4EB-P1 significantly decreased in response to inhibitor treatment indicating their potential as pharmacodynamic biomarkers of GDC-0941 activity [258]. Due to its outstanding therapeutical and pharmacological profile, GDC-0941 was subjected to clinical development. A series of phase I clinical trials are currently being conducted [259]. Initial results from a phase I study evaluating the pharmacokinetics (PK) and pharmacodynamics (PD) of the drug showed that GDC-0941 is generally well tolerated and presents signs of antitumor activity. Additionaly, a biomarker analysis of target modulation consisting of a decline in pAkt levels in platelet-rich plasma and a decrease in pS6 staining in tumor tissue was positive [260, 261].

BEZ235 it is a novel and potent inhibitor belonging to the class of imidazoquinolines, which was designed based on the structure of previously identified dual PDK1/PI3K lead compounds (Table **3**) [262]. It reversibly binds to class I PI3Ks and mTOR by competing at their ATP-binding site. The reduced phosphorylation of Akt and mTOR downstream effectors, such as GSK3β, forkhead in human rhabdomyosarcoma-like 1 (FKHRL1) and S6K observed in response to BEZ235, as well as the lack of other protein kinase targets confirmed the high selectivity and specificity of the inhibitor [263]. In terms of cellular responses, it was shown that BEZ235 was able to block proliferation and induce a G1 cell cycle arrest in a panel of cancer cell lines of different origins [263]. The cell lines investigated displayed various alterations in the PI3K/Akt pathway: U87MG glioblastoma, PC3M prostate tumor cell lines exhibited loss-of-function of the *PTEN* tumor suppressor gene, whereas breast cancer cells showed mutated *PIK3CA* (E545K, H1047R) expression, loss of *PTEN*, or amplification of *HER2*. BEZ235 was able to overcome all these oncogenic mutations, which was also documented by inhibition of Akt, S6 protein and 4E-BP1 phosphorylation. In breast cancer cell lines, observable markers of apoptosis such as caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage products were described after BEZ235 administration [264], although in some other types of tumors, an apoptotic response was not induced [265]. It was recently reported that BEZ235 treatment did not trigger cell death in breast cancer cells presenting loss of *PTEN* function or *KRAS* mutations, which was attributed to ERK pathway activation, but induced apoptosis in cells harbouring *HER2* and/or *PIK3CA* mutations [266]. The anti-proliferative activity of the compound translated very well in *in vivo* *PTEN*-null U87MG and PC3M tumor xenografts, which displayed suppression of tumor growth after oral administration of the drug [263]. The same response was observed in

Table 3. Phosphatidylinositol 3-Kinase (PI3K) Inhibitors in Preclinical and Clinical Development, their Targets and Therapeutical Application

Inhibitor	Targets	Developmental Stage	Therapeutic Application	Refs.
Pan-PI3K inhibitors				
Wortmannin	Class I PI3K, mTOR, DNA-PK, PLK1	Preclinical	Cancer	[307-311]
LY294002	Class I PI3K, mTOR, DNA-PK, ATM, ATR, p97/VCP, PXDK, CK2	Preclinical	Cancer	[309, 312-314]
SF1126	Class I PI3K, mTOR, DNA-PK, PIM1, PLK1, CK2	Phase I	Cancer	[248-251]
GDC-0941	Class I PI3K	Phase I trial	Cancer	[256-261]
BKM120	Class I PI3K	Phase I/II trial	Cancer	[274, 276, 277]
XL147	Class I PI3K	Phase I/II trial	Cancer	[278-280, 283, 284]
ZSTK474	Class I PI3K	Preclinical	Cancer	[294-298]
PX-866	Class I PI3K	Phase I trial	Cancer	[247, 315]
Dual pan-PI3K/mTOR inhibitors				
PI-103	Class I PI3K, mTOR, DNA-PK	Preclinical	Cancer	[252-255]
BEZ235	Class I PI3K, mTOR	Phase I/II trial	Cancer	[262-273]
BGT226	Class I PI3K, mTOR	Phase I/II trial	Cancer	[274, 275]
XL765	Class I PI3K, mTOR, DNA-PK	Phase I/II trial	Cancer	[281, 282, 285]
Isoform-specific PI3K inhibitors				
CAL-101	p110δ	Phase I	Cancer, allergic rhinitis	[286-291]
IC87114	p110δ	Preclinical	Cancer, allergic asthma, RA	[193, 198, 292, 293, 316, 317]
TG100-115	p110δ p110γ	Phase I/II trial	MI ischemic damage, asthma, COPD	[203, 213, 318]
AS-605240	p110γ	Preclinical	RA, SLE, glomerulonephritis, atherosclerosis	[187], [191], [209]
TGX-221	p110β	Preclinical	Arterial thrombosis	[232]
PtdIns(3,4,5)P_3 analogues				
2-O-Bn-InsP5	PH-domain bearing proteins (e.g. Akt, PDK1)	Preclinical	Cancer	[305]

Information about clinical trials status was retrieved from [306].

a xenograft mouse model of BT474 breast cancer cells harbouring either the H1047R hotspot mutation in *PIK3CA* or the empty vector, where H1047R-expressing tumors responded even better then mock control [264]. BEZ235 was also described as a potent anti-angiogenic drug. It inhibited VEGF-induced endothelial cell proliferation *in vitro* and *in vivo*. In nude mouse models the compound caused an efficient elimination of VEGF-induced vasculature leakage in the normal and tumor tissue environment and led to a strong reduction in Akt and S6 protein phosphorylation levels [267]. Additional reports showed that BEZ235 affects cell proliferation, tumor growth, adhesion, migration and metastasis in the most common musculoskeletal sarcomas such as Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma [265]. The drug's antitumor activity against multiple myeloma (MM) was also reported [268, 269]. Recent studies presented BEZ235-dependent inhibition of tumor growth in orthotopic mouse models of pancreatic cancers which is considered as a type of cancer that is rather aggressive and highly resistant to chemotherapeutics and radiation [270]. In addition, BEZ235 was much more efficient in suppressing tumor growth in renal cell carcinoma (RCC) xenografts than was rapamycin [271]. Due to its high efficacy in cancer treatment and all necessary properties required for clinical development, BEZ235 is currently being investigated in phase I/II clinical trials in patients with solid tumors enriched for patients with advanced breast cancer [272]. The first reports from clinical trials conducted in patients with solid tumors showed promising drug safety and tolerability, and a high activity, especially in tumors bearing PI3K pathway alterations [273].

At the same time as the BEZ235 inhibitor was described, a similar oral PI3K/mTOR inhibitor BGT226 and a class I pan-PI3K BKM120 inhibitor were tested in preclinical

studies [274]. They both demonstrated equally good anti-proliferative activity against tumor cell lines in animal models of cancer, as did BEZ235. Currently they are being investigated in phase I/II clinical trials in solid tumors [275, 276]. Initial reports from a phase I study of BKM120 in patients with advanced solid tumors showed anti-tumor activity [277]. There are no informations available so far concerning the clinical evaluation of BGT226.

XL147 and XL765 are two ATP-competitive PI3K inhibitors which have entered phase I/II clinical trials in recent years (Table **3**). In addition, XL147 inhibits the extracellular signal-regulated kinase (ERK) pathway by inducing a reduction in pERK and pMEK [278], but does not affect the kinase activity of Vps34, DNA-PK or mTOR [279, 280]. In contrast, XL765 is also active against mTOR [281, 282]. Both orally administrated agents inhibited tumor growth and proliferation, as well as angiogenesis, and induced apoptosis in various xenograft models bearing PI3K pathway-activating mutations [278-282]. Moreover, in cellular settings, XL147 blocked hepatocyte growth factor (HGF)-stimulated cell migration [279]. Several clinical trials are currently being conducted for both inhibitors [283-285].

CAL-101 is a new, potent p110δ-specific inhibitor which exhibits 40- to 300-fold selectivity for the p110δ isoform, as compared to other PI3K enzymes [286]. Since p110δ is primarily expressed in hematopoietic lineages preclinical evaluation of the drug in MM and CLL resulted in very encouraging results, which gave a strong basis for clinical studies [287, 288]. Treatment of p110δ-positive MM cell lines and patients samples with the inhibitor induced cytotoxic effects and cleavage of caspase-3, 8, 9 and PARP, suggesting that CAL-101-dependent cytotoxicity is driven through both the intrinsic and the extrinsic apoptotic pathways [287]. The cytotoxic response after drug treatment was not triggered in peripherial blood minonuclear cells originating from healthy donors. CAL-101-mediated concomitant suppression of Akt and Erk1/2 as well as PDK1 phosphorylation, which correlated with the induction of autophagy at earlier time point than the initiation of apoptosis. The inhibitor also turned out to be effective in overcoming drug-resistance in MM, as well as proliferative and anti-apoptotic signals mediated by IL-6 and IGF-1, which are secreted by co-cultured bone marrow stem cells (BMSCs). Moreover, capillary-like tubule formation, as well as phosphorylation and expression of Akt and Erk were also suppressed in HuVEC cells [287]. In other recently published studies on CLL cell lines and patient samples CAL-101 showed an equally good efficacy. It demonstrated an ability to act independently of CLL mutational prognostic markers (del (17p.13.1 and IgV_H) and, in contrast to other CLL agents, showed a lack of off-target toxic effects in non-hematopoetic cells [288]. Due to these very promising results, CAL-101 has been proposed for clinical studies in hematologic malignancies. A phase I trial on relapsed or refractory hematological malignancies is currently ongoing in CLL, AML, MM and non-hodgkin lymphoma (NHL) patients [289]. First interim reports from phase I trials with CAL-101 showed promising drug activity and a lack of severe toxicity in hematological cancer patients [286, 290]. Taking into account that p110δ is involved in the immune response, CAL-101 was also tested in subjects with allergic rhinitis [291].

In addition to the second generation inhibitors which have successfully entered clinical development there are other compounds, which have shown very good results in preclinical studies of cancer treatment, but were not considered for clinical trials.

IC87114 is one of the first generation inhibitors descriminating among class I isoforms. The drug selectively targets the p110δ enzyme and was identified in a chemical library screen for the determination of PI3K involved in leukocyte migration. It exhibits 58-fold selectivity for p110δ over p110γ and over 100-fold selectivity towards p110α and p110β. In addition, IC87114 did not inhibit the activity of other protein kinases, which supports its specificity [292]. In terms of biological responses, IC87114 was originally presented as an inhibitor of fMLP-stimulated $PtdIns(3,4,5)P_3$ production and neutrophil chemotaxis. It especially affected neutrophil polarization and directional migration, which emphasized a significant and selective role of p110δ in chemotaxis [292]. In the context of human cancer, IC87114 was shown to be effective in primary AML cells, which present much higher expression levels of p110δ isoform than other enzymes of class I PI3K. The drug inhibited p110δ-regulated phosphorylation of Akt and in turn AML cell proliferation [293]. IC87114 treatment of normal haematopoietic $CD34^+$ progenitor cells did not induce cytotoxic effects, which is of great advantage for normal haematopoiesis *in vivo* [293]. These studies not only shed light on the importance of p110δ in AML pathogenesis, but also underlined the therapeutic potential of selective inhibition of single PI3K isoform in contrast to blockade of all class I PI3Ks.

ZSTK474 was identified based on its strong anti-proliferative activity in a chemical library screen of s-triazine derivatives [294]. In contrast to other well-known PI3K inhibitors, the drug presented higher specificity and potency for PI3K and no activity against other tested protein kinases such as mTOR [295] and DNA-PK [296]. Although it is considered as an ATP-competitive pan-PI3K inhibitor, ZSTK474 demonstrated a slightly higher potency towards the p110δ isoform [295]. It inhibited the phosphorylation of Akt *in vitro* and *in vivo*, as well as the phosphorylation of its downstream effectors such as GSK-3β, FKHRL1, FKHR, Tuberous sclerosis 2 protein (TSC-2), mTOR and S6K. However, the Ras/Erk pathway was not affected by inhibitor treatment. When orally administrated, the compound caused a strong suppression of tumor growth *in vivo* without induction of severe toxicity [294]. Instead of promoting apoptosis in cancer cells, ZSTK474 induced a strong G0/G1 arrest and impaired proliferation through a reduction of proliferation marker expression, such as nuclear cyclin D1 and Ki67 [294, 297]. Moreover, its anti-angiogenic activity *in vivo* was demonstrated [298].

Beside applying isoform-specific or pan-PI3K inhibitors an alternative approach employing inositol polyphosphates (IPPs) has been proposed to interfere with PI3K/Akt pathway in cancer [299]. An exogenous inositol 1,3,4,5,6-pentakisphosphate (InsP5) was used to block Akt phosphorylation and in turn promote apoptosis in human cancer cells [300], as well as to prevent tumor progression and angiogenesis in *in vitro* and *in vivo* settings [301, 302]. InsP5 is a water-soluble compound, naturally occuring in mammalian cells

[303] and in some types of beans and nuts [304]. It is quickly absorbed by the cells and very stable in the intracellular environment [302]. Similarly to PtdIns(3,4,5)P_3 in the plasma membrane, InsP5 recruits and binds to PH-domains of different effector molecules such as Akt and prevents their activation by blocking translocation to the plasma membrane [301, 302]. Recently, an improved version of InsP5 was synthesised by addition of a benzyl group to InsP5 [305]. 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP5) not only blocked Akt translocation to the membrane, but also selectively inhibited PDK-1-dependent phosphorylation of Akt. It showed much higher potency against PDK-1 than its precursor InsP5, and additionally it specifically suppressed mTOR *in vitro* [305]. Moreover, 2-O-Bn-InsP5 displayed an enhanced pro-apoptotic activity in cancer cell lines and an augmented ability to reduce tumor growth in xenograft models which were resistant to InsP5 treatment. Furthermore, 2-O-Bn-InsP5 was able to induce cell death even in usually chemotherapy-resistant pancreatic cancer cells [305]. Although 2-O-Bn-InsP5 does not directly inhibit any of the PI3K isoforms, it mimics the signaling function of PtdIns(3,4,5)P_3 and blocks signal transduction through the PI3K/Akt pathway. Exploiting the ability of 2-O-Bn-InsP5 to recruit PH-domains-bearing proteins is an interesting example of a potential novel anti-cancer approach.

5.2. PI3K Inhibitors in other Diseases

While p110α plays an important function in oncogenesis, p110β, p110δ, and p110γ are essential for thrombosis, immune system function, and inflammation, respectively. The involvement of PI3K enzymes in the pathogenesis of other human diseases, such as atherosclerosis, chronic inflammation, allergy, autoimmunity, cardiovascular and metabolic disorders, opened an area for the development of additional PI3Ks inhibitors. An example of such a drug exhibiting broad anti-inflammatory activities is TG100-115 (Table **3**) [213]. It was shown to potently inhibit vascular endothelial growth factor (VEGF) signaling which triggers edema and the inflammatory response which can also be induced by a wide number of other ligands upstream of RTKs and GPCRs. Importantly, TG100-115 did not block VEGF-driven angiogenesis *in vivo* and VEGF-stimulated ERK activation, which is a signaling event responsible for the mitogenic response to this growth factor [213]. As a result, the mitogenesis of endothelial cell was not perturbed, and a tissue repair process, which is critical for tissue survival after ischemic damage in MI could arise. Two known proinflamatory mediators, namely p110δ and p110γ, turned out to be the targets of TG100-115 in the context of inflammation-associated aspects of VEGF signaling. These enzymes were both inhibited with a much higher potency than other members of class I PI3Ks and a wide range of other protein kinases. The excellent specificity of the inhibitor was proven by conformational rotation studies. It turned out that the greater the barrier to rotation an inhibitor has, the more isoform-selective it is. This was the case for TG100-115 in comparison to other pan-PI3K agents [213]. Its prominent selectivity was accompanied by extreme effectiveness *in vivo*. The compound reduced infarct development and improved myocardial function in aggressive rodent and porcine MI models. Cardioprotection was achieved after delivering the drug at the time period when MI patients are most accessible for therapeutic intervention (up to 3 hours). This and other criteria such as a high efficacy at relatively low doses and single administration, long-lasting effects of p110δ/γ inhibition and the intravenous route of drug delivery appeared to be very beneficial for clinical use. TG100-115 is the first small-molecule kinase inhibitor which was studied in acute MI patients when delivered after ischemic damage and also one of the first isoform-specific PI3K inhibitors which has entered clinical trials [213]. Phase I and II clinical trials, aiming at an evaluation of TG100-115 safety and potential efficacy in the restoration of blood flow and the reduction of heart muscle damage after heart attack in MI patients was completed in 2008 [318]. The anti-inflammatory potential of TG100-115 was also tested for asthma and chronic obstructive pulmonary disease (COPD) [203]. The favorable pharmacokinetics, safety, and biological activity of TG100-115 were shown with the use of mouse models of these respiratory diseases [203].

Another specific PI3K inhibitor which is considered as a promising candidate for the treatment of inflammatory and autoimmune disorders is termed AS-605240. It blocks p110γ catalytic activity downstream of GPCRs, in an ATP-competitive manner and is active in RA [191]. p110$\gamma^{-/-}$ mouse models displayed defects in neutrophil migration and were protected against RA. Oral administration of the drug to wild type mice (p110$\gamma^{+/+}$) mimicked the effect of p110γ deficiency and minimized the progression of joint inflammation [191]. Furthermore, AS-605240 was able to overcome SLE development in SLE-prone MRL-*lpr* mice without inducing toxic effects during the treatment period (see section 4.2.1) [187]. In addition, different stages of atherosclerosis progression in mouse models were also affected by the inhibitor treatment [209]. Thus, targeting p110γ with this inhibitor seems to be very promising for the treatment of chronic inflammatory disorders.

Inhibition of p110δ with the use of IC87114 appeared to be very effective in reducing the symptoms of allergic asthma [198, 317]. Administration of the drug to the OVA-induced mouse models of asthma substantially repressed airway inflammation, airway hyperresponsiveness and T_h2 cytokine levels. This effect was achieved due to p110δ-dependent suppression of intereukin-17 (IL-17) expression, which was mediated by changes in Akt-induced NF-κB activation [198]. Besides asthma, the p110δ-selctive IC87114 agent was also shown to significantly suppress the bone and cartilage destruction typical for RA in wild type mice (see section 4.2.1) [193]. However, bone and cartilage damage was completely absent when IC87114 was administrated to p110$\gamma^{-/-}$ mice, suggesting the therapeutic potential of IC87114 as an adjuvant therapy for RA.

The fact that all class I PI3K isoforms are expressed in platelets has caused an increased interest in understanding the possible role of the enzymes in the progression of cardiovascular diseases. In turn, this area of human medicine has also benefited from the development of PI3K-targeting therapeutic strategies. The discovery that p110β is involved in the process of pathological thrombosis resulted in the identification of the TGX-221 inhibitor, which protected mouse models against occlusive thrombus formation [232].

In contrast to other antithrombotic drugs (e.g. aspirin, clopidogrel), TGX-221 did not affect platelet homeostasis and thus did not induce bleeding complications. Animal models of arterial thrombosis showed normal tail bleeding time. This feature is undoubtedly of big advantage in terms of a possible utilization of the drug in the clinic. TGX-221 is a cell-permeable analogue of LY294002, exhibiting a high potency in the low nanomolar range and a specificity for p110β. It exhibits ~ 1000-fold selectivity for p110β over p110α and p110γ, and > 1000-fold selectivity over a broad range of protein kinases [232]. By inhibiting p110β under high shear stress conditions, TGX-221 abolished the cytosolic calcium flux and integrin $\alpha IIb\beta_3$ activation in response to ADP and treshold doses of other soluble agonists (triiodothyronine receptor auxiliary protein, epinephrine). Thus, shear stress-induced PtdIns(3,4)P_2 generation and integrin $\alpha IIb\beta_3$-mediated stable adhesion contacts in platelets were blocked. Beside defining a new role for p110β in integrin $\alpha IIb\beta_3$-dependent stable platelet adhesion and aggregation, these studies identified a new isoform-selective PI3K inhibitor which appears to exhibit antithrombotic potential [232].

5.3. Perspectives for PI3K-Targeting Agents in Overcoming the Problem of Drug Resistance in the PI3K/Akt/mTOR Pathway

Drug resistance is a complicated concept which involves many aspects of human biology. First of all, it is differentiated into primary and acquired resistance mechanisms. In addition, multiple crosstalks between signal transduction pathways, feedback loops, multiple genetic alterations, such as secondary mutations in the drug target or in parallel pathways, and recently reported dynamic chromatin modifications make it difficult to unravel and translate new discoveries to the clinic [319, 320]. However, there are some facts about drug resistance mechanisms which are already known, especially in human cancer treatment. The PI3K family of enzymes represents one of the pieces of the puzzle of drug resistance.

5.3.1. Oncogenic Mutations

Continuous PI3K/Akt signaling is a major cause of resistance to anticancer drugs. Enhanced activation of the pathway is induced through genetic alterations such as *PIK3CA*, *PIK3R1* and *AKT*-activating mutations, amplifications of *PIK3CA*, RTKs (e.g. *EGFR* family) and *AKT*, or *PTEN* loss-of-function (Table **2**). The coexistence of these oncogenic abnormalities [171], or point mutations in the drug affinity pocket of PI3K, which diminish effective drug interaction and block the enzymes [172] also contribute to drug resistance. It is known that mutations in the most important components of the PI3K/Akt axis (e.g. *PIK3CA*, *PTEN*, *PIK3R1*) lead to oncogenic activity of the enzymes and result in resistance to chemotherapeutics, as well as therapeutic agents directed against other components of the pathway (e.g. EGFR family) [171, 173]. Breast cancer patients subjected to therapy with the anti-HER2 antibody trastuzumab developed resistance and were characterized as patients with poor prognosis due to hyperactivation of the PI3K pathway [171, 173]. It was described that in breast cancer cells carrying amplified *HER2* receptor, p110α-activating mutations (E545K, H1047R) confer resistance to trastuzumab [264]. Treatment of these cell lines with BEZ-235 inhibited proliferation and hyperactivation of the PI3K/mTOR pathway induced by *PIK3CA* mutations, which was reflected by reductions in Akt, S6 and 4EPB1 phosphorylation. The tumor growth in trastuzumab-resistant breast cancer xenograft models was also suppressed [264].

A different example of an inhibitor overcoming drug resistance is PX-866, an analogue of wortmannin. It acts on the p110α, γ and δ isoforms of class I PI3K and shows decreased potency against p110β and mTOR [247, 315]. The majority of non–small cell lung cancer (NSCLC) patients bearing EGFR mutations respond well to EGFR inhibitors, such as gefitinib. Furthermore, gefitinib inhibits activation of the PI3K signaling pathway due to the blockade of EGFR and ErbB-3 receptor dimerization after stimulation with ligand. A-549 NSCLC tumor xenografts, which do not express ErbB-3, turned out to be resistant to gefitinib. Treatment of these tumors with low doses of PX-866 prior to gefitinib administration potentiated the antitumor activity of the latter compound and overcame resistance. Full growth control and a reduction in Akt phosphorylation was achieved, which was not observed when xenografts were treated with gefitinib alone [315]. Although PX-866 showed some toxic effects, such as decreased glucose tolerance and increased neutrophil counts after prolonged administration, this did not disqualify it as a candidate for clinical use. Decreased glucose tolerance was abolished upon treatment cessation or reversed by insulin and by the peroxisome proliferator-activated receptor-γ (PPAR-γ) activator pioglitazone. PX-866 is the only irreversible PI3K inhibitor which has entered the clinical stage of studies [247, 315]. A final result from the phase I clinical trial of orally administrated PX-866 showed inhibition of PI3K pathway as well as the drug's tolerability, which supported phase II testing [321].

5.3.2. Feedback Loops

Negative feedback loops are one of the causes for the development of drug resistance. The best known example of such a regulation involves the inhibition of mTOR with rapamycin which causes an activation of the Akt [322] and/or ERK/MAPK pathway [323] promoting pro-survival and proliferative signaling in different types of tumors (refer to Fig. **1**). Suppression of mTOR with rapamycin inhibits S6K, resulting in up-regulation of IRS-1 protein level and activation of the IGF-IR/IRS-1/PI3K signal transduction pathway, which in turn leads to Akt phosphorylation [322]. On the other hand, the activation of the RTK/IRS-1/PI3K axis can be directed towards the Ras/MAPK pathway followed by Akt and Erk phosphorylation and activation of downstream signaling which attenuate the anti-proliferative and anti-apoptotic effects of rapamycin [323]. This dual feedback mechanism can be avoided by using a combinatorial treatment of an mTOR inhibitor with growth factor receptor, MEK or PI3K inhibitors. Dual PI3K/mTOR inhibitors also appear to fulfil the required criteria. As suggested by Manara *et al.* or Maira *et al.* BEZ235 might be a good candidate for this application [263, 265].

Although examples of overcoming protein kinase drug resistance by PI3K inhibitors are described adequately, we cannot forget that the resistance to PI3K inhibitors can also occur. However, the mechanism is likely to be different from

that of the protein kinase family (e.g. BCR-Abl, c-Kit, PDGFR, EGFR) in which it is driven by the point mutation of the "gatekeeper" residue located at the affinity pocket, where the most potent inhibitors bind. Analogously to other protein kinases, mutation of the Ile848 residue was proposed to govern drug resistance in class I PI3Ks. Interestingly, prediction studies of possible drug-resistance mutations in p110α identified other residue than Ile848, namely Ile800, which accounts for 5-30 fold resistance to most PI3K inhibitors. However, additional studies are required to draw a full picture of resistant mutations in p110α [172].

5.4. PI3Ks Structural Studies Impact on Drugs Design

Structural characterization of the PI3K isoforms in both free form or in complex with regulatory subunits or ATP-competitive small molecule inhibitors shed a new light on the drug discovery programs (extensively discussed in [324]). Improvement of the specificity and potency of the anti-cancer drugs is currently the main driving force of the PI3Ks structure elucidation. Employment of more selective compounds in the clinic avoids undesirable phenotypes and toxic side effects which are usually induced by pan-class I PI3K inhibitors.

The ATP-binding pockets of class I PI3Ks are highly conserved [314]. Therefore, it became an important task to determine structural differences among the isoforms which can be utilized for designing more specific drugs. For a long time class I p110γ was the only PI3K isoform whose structure had been described [325]. Later on, a full-length structure of the most frequently mutated PI3K isoform in human cancer, p110*α*, was characterized in a complex with the N-terminal-SH2 (nSH2) and inter-SH2 (iSH2) domains of the p85*α* subunit [149]. These studies also provided new insights into the location of some of the oncogenic mutations in p110α that is of a great value for designing mutant-specific inhibitors [326]. The recent publication of p110δ structure uncovered new structural determinants which control selectivity across PI3Ks [327]. Detailed analysis of the p110δ ATP-binding site organization when occupied by small molecule inhibitors revealed that the selectivity toward the enzyme is driven by its conformational flexibility and the sequence diversity of active site residues that are located away from the direct ATP binding pocket. The availability of this information resulted in the synthesis highly specific inhibitors for p110δ with greatly improved potencies [327]. The advantages derived from structure-based drug design are therefore convincing and encouraging further investigations.

5.5. Combinatorial Treatments

Using small molecule PI3K inhibitors as single agents for the effective treatment of different human diseases may not always bring substantial and long-lasting benefits. Development of acquired resistance including the induction of multiple secondary mutations or activation of the substituting kinases, and feedback loops resulting from numerous crosstalks between different pathways, make this task even more difficult. A growing understanding of the complex interactions between signaling networks has led to the development of rationally designed, effective combination therapies. Numerous preclinical investigations documenting the effectiveness of combinations of PI3K inhibitors with other therapeutic strategies (including conventional chemotherapy and radiation) are already published and are being investigated in the clinics (reviewed in [328]). Recent reports have indicated that a combination of GDC-091 with the anti-HER2-directed antibody trastuzumab or pertuzumab decreased the growth of *HER-2*-amplified (trastuzumab-resistant) breast cancer cells [329]. The BEZ235 inhibitor significantly enhanced the efficacy of temozolomide in *PTEN*-null glioblastoma multiforme mouse model by causing a regression of the tumor mass [263]. BEZ235 was also suggested to be used with trastuzumab for treatment of breast cancer patients with *HER2* and/or *PIK3CA* mutations [264, 266], or in combination with MEK inhibitors for *PTEN*- or *KRAS*-mutated breast and lung cancers, respectively [266, 330]. These and plenty of other examples including SF1126 [249], IC87114 [293] and CAL-101 [287] combinatorial treatments with taxotere, VP16 (a topoisomerase II inhibitor) and bortezomib respectively, provided a proof-of-concept for the vertical and horizontal blockade of crosslinked signaling pathways, as proposed by Yap *et al.* [331] and Workman *et al.* [324]. The vertical blockade is especially effective in preventing secondary drug resistance driven by negative feedback loops. It involves one or more drugs which target the same signaling pathway at different levels, such as dual PI3K/mTOR inhibitors (e.g. BEZ235), or selective EGFR and PI3K inhibitors combined together [264]. The concept of using multi-targeted single agents or cocktails of highly selective inhibitors was described by Knight *et al.*, [332] and supported by Zunder *et al.* [172], who suggest that multi-targeted drugs of PI3K pathway (e.g. PI-103 and BEZ-235) are less likely to cause drug resistance than selective PI3K inhibitors. On the other hand, PI3K and MEK inhibitors can serve as an example of the horizontal way of suppression of molecular targets, which involves the use of drugs which inactivate two or more different parallel signaling pathways [330, 333]. However, besides the many advantages derived from combinatorial strategies they have also raised many questions and concerns about the toxicity of such treatments (reviewed in [328]). There is always a risk of developing undesired effects resulting from disturbances in the homeostasis in healthy cells. Therefore, a careful analysis of previous results is needed to design the best combinatorial approaches.

In addition, an early selection of patients who would benefit form the combination therapy is crucial for an effective treatment. Even though we have nowadays acquired an increased knowledge from structural inhibitor studies, mouse models and clinical trials, it is still a big challenge to select patients who will benefit the most from a specific treatment. In the future, biomarkers will become a major aspect for developing successful treatments, and our growing comprehension of the molecular map of human cancer is giving us the hope that this will soon be possible.

6. CONCLUSIONS

The ubiquitous expression of PI3Ks in different cells, tissues and organs underscores their significance and multiple functions in an organism. In connection with others signal transduction pathways, the PI3K/Akt axis forms a complex signaling network controlling a wide variety of biological processes. Deregulation of the PI3K/Akt pathway

results in a disruption of cellular homeostasis, which further leads to different pathological states reflected in the development of various human diseases. Accordingly, the PI3K enzymes have become promising targets for therapeutic intervention in cancer, as well as inflammatory, autoimmune and cardiovascular disorders. Small molecule inhibitors targeting PI3K isoforms have evolved over the years from natural, but quite toxic and unstable compounds to better tolerated and more potent synthetic agents. These inhibitors have become a major force in the arsenal of anticancer therapies, as well as potential new drugs for the treatment of other human diseases. As known from numerous preclinical studies and the first reports from clinical trials, PI3K inhibitors may work efficiently as single agents, but also in combination with other chemotherapeutics. The future choice of a clinical approach involving these inhibitors will strongly depend on the genetic background of the patients and, therefore, biomarker profiles will be essential in predicting which patients will most likely benefit from a specific agent. The availability of such information will be of great value for the design of more personalized therapies.

The translation of the increasing knowledge about PI3K signaling to clinical settings is a challenging task for the future. Fortunately, an increasing number of encouraging reports from the first clinical trials give hope that this goal is achievable.

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ABBREVIATIONS

2-O-Bn-InsP5	=	2-*O*-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate
4E-BP	=	Eukaryotic translation initiation factor 4E-binding protein 1
p97/VCP	=	p97/valosin-containing protein
ALL	=	Acute lymphoid leukaemia
AML	=	Acute myeloid leukaemia
CLL	=	Chronic lymphocytic leukemia
DNA-PK	=	DNA-dependent protein kinase
EGFR	=	Epidermal growth factor receptor
ErbB-3	=	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
ERK	=	Extracellular signal-regulated kinase
FKHR	=	Forkhead human rhabdomyosarcoma transcription factor
fMLP	=	formyl-methionyl-leucyl-phenylalanine
GLUT-4	=	Glucose transporter type 4
GPCR	=	G protein-coupled receptor
Grb2	=	Growth factor receptor-bound protein 2
GSK3β	=	Glycogen synthase kinase 3 beta
HER2	=	Human epidermal growth factor receptor 2
IGF1	=	Insulin-like growth factor 1
IGF-1R	=	Insulin-like growth factor-1 receptor
IL	=	Intereukin
IRS	=	Insulin receptor substrate
LOH	=	Loss of heterozygosity
MAPK	=	Mitogen-activated protein kinase
MI	=	Myocardial infarction
MM	=	Multiple myeloma
mTORC1	=	Mamalian target of rapamycin complex 1
mTORC2	=	Mamalian target of rapamycin compelx 2
PDK1	=	3-phosphoinositide-dependent protein kinase 1
PI3K	=	Phosphatidylinositol 3-kinase, PI3-kinase
PIK3CA	=	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIK3CB	=	Phosphoinositide-3-kinase, catalytic, beta polypeptide
PIP3	=	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns	=	Phosphatidylinositol
PtdIns(3)P	=	Phosphatidylinositol-3-monophosphate
PtdIns(3,4,5)P_3	=	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4,5)P_2	=	Phosphatidylinositol-4,5-bisphosphate
PTEN	=	Phosphatase and tensin homologue deleted on chromosome 10
PXDK	=	Pyridoxal kinase
RA	=	Rheumatoid arthritis
Ras	=	Ras guanine nucleotide exchange factor 2
RGD	=	Arginine–glycine–aspartic acid targeting moiety
RTK	=	Receptor tyrosine kinase
S6K	=	p70 ribosomal S6 kinase; ribosomal protein S6 kinase, 70kDa, polypeptide 1
SLE	=	Systemic lupus erythematosus
Vps34	=	Vacuolar protein-sorting defective 34

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